# PERTURBATIONS TO THE INTERSYSTEM CROSSING OF PROFLAVIN UPON BINDING TO DNA AND POLY d(A-IU) FROM TRIPLET-DELAYED EMISSION SPECTROSCOPY

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ABSTRACT The steady-state prompt fluorescence, phosphorescence and delayed fluorescence spectra and triplet lifetimes of free proflavin and proflavin bound to native DNA and alternating poly d(A-IU) were obtained as a function of temperature in a buffer-glycerol solvent. The intensity of the proflavin E-type delayed fluorescence (DF) relative to both the phosphorescence (Ph) and the prompt fluorescence (F) was observed to increase with temperature, and plots of both *ln* (DF/Ph) and *ln* (DF/(F · $\tau_T$)) as a function of 1/T were linear over a wide range of temperatures. Although the activation energies for the thermal repopulation of the proflavin excited singlet state from the triplet obtained from the slopes of these plots were essentially unchanged on binding, perturbations to the $S_1 \rightarrow T_1$ intersystem crossing rate constants extracted from the intercepts at infinite temperature were observed. The marked enhancement of the intersystem crossing that occurs with binding to the iodinated polynucleotide reflects an external heavy atom perturbation upon the intercalated dye which also induces a shortening in the triplet lifetime. With proflavin bound to DNA an enhancement to the $S_1 \rightarrow T_1$ intersystem crossing, though lesser in magnitude than for poly d(A-IU), is observed but with no change to the triplet lifetime. The well-studied fluorescence quenching of DNA-bound proflavin is a result of this increase in the intersystem crossing. It is proposed that these non–heavy atom enhancements in the intersystem crossing are due to distortions of the molecular plane of the bound proflavin molecule. In total these analyses provide a complete description of the excited state processes of the proflavin molecule and their variations with temperature.

## INTRODUCTION

Triplet state spectroscopy of aromatic molecules provides a perspective on the structure and dynamics of macromolecules and macromolecular assemblies that is significantly informative and different than that derived from fluorescence measurements to warrant increasing interest. Because the excited triplet and singlet states are interconnected, characterization of excited-state processes will of necessity involve both states. For example, heavy atom perturbations which enhance intersystem crossing rates influence the lifetimes of both the excited singlet and triplet states as well as their relative contributions to the emission (McGlynn et al., 1969).

The excited-state processes within aromatic probes are determined by both the inherent characteristics of the particular molecule as well as the structure and dynamics provided by the macromolecular environment. Even the actual states from which the long-lived triplet emission occurs will be dependent upon a combination of intrinsic properties and environmental factors. The triplet state emission of tryptophan-containing proteins at temperatures ranging from 77K to room temperature (Saviotti and Galley, 1974) is exclusively a direct triplet-to-ground-singlet-state transition, i.e., phosphorescence. In other cases naturally occurring chromophores such as porphyrins (Parker and Joyce, 1967) and extrinsic probes such as proflavin (Parker and Joyce, 1973, Geacintov et al., 1981; Corin and Jovin, 1985) display long-lived triplet emission which at low temperature is phosphorescence and at room temperature is delayed fluorescence. These forms of delayed emission respond to perturbations such as the presence of heavy atoms in distinctive ways.

There are a number of mechanisms which give rise to the appearance of delayed fluorescence in organic chromophores, and it is necessary to establish the nature of the process under observation. With chromophores in which the energy gap between the excited singlet and triplet state is small, delayed fluorescence arising from thermal repopulation of the excited singlet state from the triplet is often observed. This type of emission was studied in some detail by Parker and Hatchard (1961, 1962) who referred to it as eosin-type, or E-type, delayed fluorescence. P-type delayed fluorescence also characterized by these authors arises from triplet–triplet annihilation requiring free diffusion or the proximity of excited triplet molecules (Parker and Hatchard, 1963). Both of the above types of emission have

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been observed with proflavin (Parker, 1964; Galley, 1967; Ewald et al., 1970). Proflavin delayed fluorescence has also been observed as a result of triplet–singlet energy transfer from tryptophan in chymotrypsin (Galley and Stryer, 1969) as well as in DNA (Isenberg et al., 1964). Finally, with excitation at higher energies delayed fluorescence from proflavin occurs due to a photoionization-recombination mechanism (Ewald et al., 1968). Despite the variety of potential mechanisms involved it is possible to distinguish them. In the present study we are concerned with the E-type (sometimes referred to as delayed thermal fluorescence) and the phosphorescence, from proflavin and the manner in which they respond to binding to nucleic acids in the absence and presence of heavy atoms.

The majority of biophysical studies involving triplet states have involved either observations in rigid media at 77K or in solution at ambient temperatures. We have found it useful to continuously follow the emission properties as a function of temperature from the low-temperature rigid glass limit to room temperature. Using glycol-water solvents this approach has proved useful in establishing limits for the emission intensities and the triplet lifetimes in proteins (Domanus et al., 1980) and in revealing spectral and lifetime heterogeneity with a given emitting species (Purkey and Galley, 1970). In addition it also allows dynamic processes, which at a given temperature might span 10 orders of magnitude, to manifest themselves (Galley and Purkey, 1970; Strambini and Galley, 1976). Such an approach was essential in the present study to monitor the temperature dependence of the relative efficiencies of the radiative pathway for the photons originating from the triplet state of proflavin, with increasing thermal energy.

Proflavin was examined in this work because it was previously known to display E-type delayed fluorescence (Parker, 1964; Geacintov et al., 1981; Corin and Jovin, 1985). The binding of proflavin to DNA has been studied at length in part due to the biological responses induced therein (Albert, 1966). Two binding forms occur at low concentrations of dye: a weak external binding form at low polymer-to-dye ratio (P/D) in which electrostatic forces play a role and a stronger binding form at higher P/D (>10) having the dye intercalated between the base pairs of the double helix (Lerman, 1961 and 1963). The presence of heavy atoms such as bromine or iodine in the vicinity of an aromatic molecule produces the well-known heavy atom effect (McGlynn et al., 1969). In response to the spin-orbital coupling imparted by the heavy atom to the aromatic chromophore, the rates of transitions between singlet and triplet states are increased. The result is a quenching of the fluorescence intensity accompanied by an increase in the quantum efficiency for intersystem crossing from the singlet to the triplet state and a decrease in the triplet lifetime. In earlier work this effect was employed as a probe of the geometry of acridine-nucleic acid complexes using bromine as the heavy atom (Galley and Purkey, 1972). In this work the alternating polynucleotide poly d(A-IU) was employed. The nucleotide, 5-iododeoxyuracil, present in the polymer is an analogue of thymine having an iodine atom instead of a methyl group in the 5 position of the pyrimidine ring. This analogue was chosen because the iodine atom induces perturbations over a somewhat larger distance than bromine (Lee, 1985).

The purpose of this work was to examine the singlet and triplet radiative pathways for the triplet state of proflavin and to observe the extent to which the emission is affected by binding to nucleic acids both in the absence and presence of external heavy atom perturbations. A kinetic analysis of the delayed emission data is presented and from this analysis a detailed description of the excited state for proflavin and the manner in which these are perturbed on binding is obtained.

## EXPERIMENTAL

DNA (calf thymus, type I), Sigma Chemical Co., St. Louis, MO; poly(deoxyadenine,5-iodo-deoxyuracil), (pdA-IU), Pharmacia P-L Biochemicals, Milwaukee, WI; proflavin, K & K Laboratories, Plainview, NY; glycerol (gold label), Aldrich Chemical Co., Milwaukee, WI; and cacodylic acid sodium salt, J. T. Baker Chemical Co., Philipsburg, NJ were used without any further purification.

Solutions were $2 \times 10^{-6}$ M proflavin in 70/30 (wt/wt) glycerol/0.02 M cacodylate buffer, pH 7.2 and where applicable $1 \times 10^{-3}$ M in DNA or pd(A-IU). The concentrations of polynucleotide were determined from the absorbance at 265 nm ($\epsilon = 6{,}600\ M^{-1}\ cm^{-1}$). All samples for emission measurements were contained in Suprasil tubes 4 mm i.d. equipped with rubber septa. Deaeration was accomplished by passing a stream of nitrogen gas over the surface of the solution for 30 min. Throughout the deaeration process the samples were cooled on ice.

The excitation light source for the emission apparatus was a 100 W high pressure mercury arc lamp (PEK, Sunnyvale, CA). The excitation wavelengths were selected by a 0.250 m grating monochromator with a dispersion of 6.0 nm/mm (Bausch & Lomb Inc., Rochester, NY). The sample emission was passed through a 0.500 m Bausch & Lomb monochromator with a dispersion of 3 nm/mm to an EMI 9635QB photomultiplier. The photomultiplier output was amplified and connected to the *y*-axis of a Mosley 7035B *xy* recorder. Triplet decays were obtained by connecting the photomultiplier output either directly to the *xy* recorder for lifetimes >0.5 s or to a Biomation 610B digital transient recorder for lifetimes <0.5 s. Where required, a pair of synchronized rotating choppers was employed to eliminate the prompt emission and excitation light. The temperature was controlled with an Oxford Instruments precision temperature controller in conjunction with a stream of precooled nitrogen. The temperature was monitored with a copper-constantin thermocouple. The temperature stability was about ±0.25°C and the accuracy of the temperature reading was about ±0.3°C.

The ratios of delayed fluorescence intensity (DF) to phosphorescence (Ph) were obtained from the delayed emission spectra. Delayed fluorescence/prompt fluorescence (DF/F) ratios could be obtained from the total emission spectra in two ways: either from the ratio of slow/prompt emission at 500 nm or from the ratio of slow/prompt emission at 570 nm in conjunction with the previously determined DF/Ph data.

At 500 nm (approximate $\lambda_{max}$ of DF) there is negligible amount of phosphorescence emission but at 570 nm (approximate $\lambda_{max}$ of Ph) the red edge of the delayed fluorescence band overlaps the phosphorescence region of the spectrum. Therefore at warmer temperatures with larger amounts of delayed fluorescence, it was necessary to correct the 570-nm delayed emission for this fact. The measured emission intensities were corrected for monochromator/photomultiplier bias, solvent contraction due to cooling and polarization where necessary (Galley, 1967). The

absorption spectra were taken on a Cary 14 spectrophotometer in 1-cm quartz cells.

## RESULTS

The excited state processes of an aromatic molecule can be described by Fig. 1. The triplet state can be observed in emission as either phosphorescence, i.e., direct emission from the triplet state, or as delayed thermal fluorescence resulting from intersystem crossing from a thermally excited triplet level back to the excited singlet state followed by singlet emission. The spectral distribution of the delayed fluorescence is essentially that of the prompt fluorescence. It was possible to characterize the delayed fluorescence as thermally induced, or Parker's E-type, from the following properties. The intensity was linear with respect to concentration of dye and to the intensity of the excitation light, ruling out two-photon processes such as triplet annihilation; the decay of the emission followed first order kinetics; at any given temperature the lifetimes of the delayed fluorescence and phosphorescence were equal and the ratios of delayed fluorescence to phosphorescence and of delayed fluorescence to prompt fluorescence had an Arrhenius type relationship with respect to temperature.

The relative amounts of delayed fluorescence and phosphorescence depend upon the triplet singlet energy gap, $\Delta E$, the temperature and the intersystem crossing efficiency, $\phi_{ix}$. In the appendix we have derived expressions for the ratios of the delayed fluorescence to phosphorescence and delayed fluorescence to prompt fluorescence which are used here to analyse the proflavin emission.

This emission of proflavin has been monitored in aqueous glycol media over a range of temperatures which provide a rigid glass at the low end of the scale and fluid solution at the warm end. In doing so it has been possible to observe the limits of the fluorescence, delayed fluorescence, and phosphorescence intensities and of the triplet lifetimes, the presence of spectral heterogeneity at low temperature and the variations in these properties upon binding to nucleic acids.

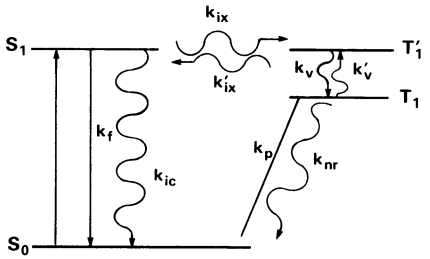


FIGURE 1 An energy level diagram for an aromatic molecule such as proflavin. $S_0$ and $S_1$ are the ground state and the lowest excited singlet state. $T_1$ is the lowest triplet state and $T_1'$ is a thermally excited vibrational level in the $T_1$ electronic state and is isoenergetic to $S_1$. The rate constants of the excited state processes are: $k_f$, fluorescence; $k_{ic}$, internal conversion of $S_1$; $k_{ix}$, intersystem crossing, assumed to be equal in the forward and reverse directions; $k_v$ and $k_v'$, the thermal transitions between $T_1$ and $T_1'$; $k_p$, phosphorescence; $k_{nr}$, nonradiative decay of $T_1$.

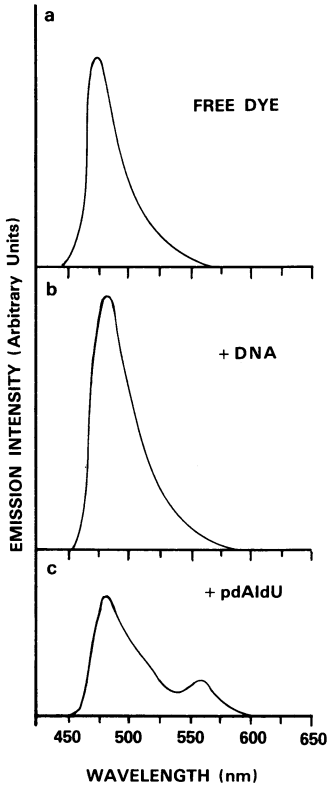


FIGURE 2 The total emission spectra of proflavin in glycerol/buffer (70/30 wt/wt) solution at 77K. (*a*) Free dye; (*b*) bound to DNA; (*c*) bound to pd(A-IU). The spectra are uncorrected for instrumental sensitivity bias.

The total emission spectrum of free proflavin, shown in Fig. 2, was predominantly prompt fluorescence, the delayed emission (phosphorescence at this temperature) making only a minor contribution. When the prompt fluorescence was eliminated with a phosphoroscope and the sensitivity increased, the delayed emission was observed. The delayed emission spectrum of proflavin is shown in Fig. 3 for two different temperatures. Below

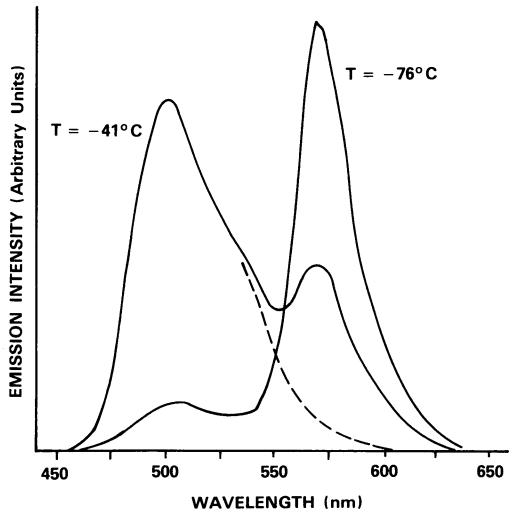


FIGURE 3 The delayed emission spectra of proflavin bound to DNA in deaerated glycerol/buffer (70/30 wt/wt) solution at −76 and −41°C. The broken line under spectrum 2 represents the delayed fluorescence component of the delayed emission at −41°C. The spectra are uncorrected for instrumental sensitivity bias.

TABLE I
QUANTUM EFFICIENCIES, LIFETIMES, AND RADIATIVE RATE CONSTANTS OF PROFLAVIN

| Dye preparation | $\phi_f$* | | | $\tau_S$‡ | $\tau_T$ | $\phi_{ix}$ | $k_p$ | $\phi_p$ | Ph/F ratio§ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | sp‖ | calc¶ |
| | | | | *ns* | *s* | | $s^{-1}$ | | | |
| Free (aq) | 0.39** | | | | | | | | | |
| Free (gl) | 0.55 | 0.85 | 0.90 | 8.9 | 0.60 | 0.099 | 0.051 | 0.031 | 0.0085 | 0.0036 |
| +DNA (aq) | 0.19 | | | | | | | | | |
| +DNA (gl) | 0.30 | 0.76 | 0.78 | 8.9 | 0.25 | 0.23 | 0.036 | 0.009 | 0.0031 | 0.0027 |
| +pdAIU (gl) | 0.12 | 0.25 | 0.31 | 2.8 | 0.11 | 0.58 | 2.4 | 0.26 | 0.39 | 0.60 |
| Temperature (°C) | 20 | −55 | −100 | −55 | −55 | −55 | −55 | −55 | −55 | |

aq, aqueous buffer; gl, 70/30 glycerol/buffer; +DNA and +pdAIdU, $1 \times 10^{-3}$ M in polynucleotide phosphate; *Lee and Galley (unpublished); ‡see Appendix; §the ratio unpublished of the phosphorescence to fluorescence intensities from ‖corrected total emission spectra and ¶predicted value; **Parker and Joyce (1973); $\phi_f$, $\phi_{ix}$, $\phi_p$ are the quantum efficiencies of fluorescence, intersystem crossing, and emission from the triplet state, respectively; $\tau_S$ and $\tau_T$ are the natural lifetimes of the singlet and triplets states, respectively; $k_p$ is the radiative rate constant of the triplet state.

−76°C the emission was almost entirely phosphorescence. As the temperature was increased the relative amount of delayed thermal fluorescence in the delayed emission spectrum increased and at temperatures above −20°C the delayed fluorescence predominated.

The binding of proflavin to DNA at polymer-to-dye ratios >500 leaves the low temperature total emission spectrum largely unchanged. A decrease in the fluorescence efficiency, consistent with previous work (Kubota et al., 1978; Georghiou, 1975) and due presumably to the presence of guanosine-cytosine pairs, was observed (see Table I). An increase in the ratios of delayed fluorescence to phosphorescence and of the delayed fluorescence to prompt fluorescence was observed. This can be seen by a comparison of the positions of the plots in Fig. 4 with those of Fig. 5 and will be discussed in greater detail below. The phosphorescence decays of the free and DNA-bound dyes were essentially the same at 77K. Both dye preparations yielded single component decays with lifetimes of 2.4 s.

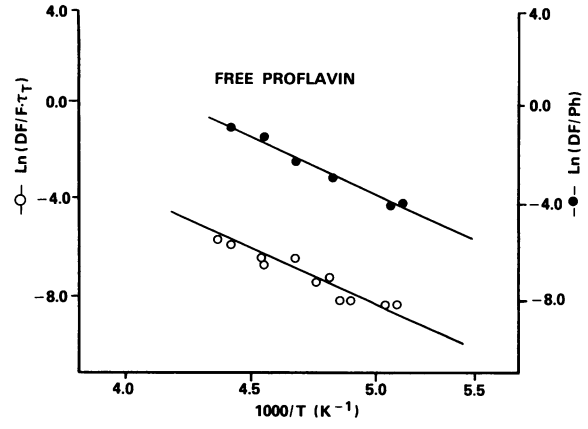


FIGURE 4 The variation of ln (DF/Ph) (closed data points) and ln (DF/F · $\tau_T$) (open data points) vs. reciprocal temperature for free proflavin in glycerol/buffer (70/30 wt/wt) solution. DF, Ph, and F represent, respectively, the corrected emission intensities for delayed thermal fluorescence, phosphorescence and prompt fluorescence; $\tau_T$ is the natural lifetime of the triplet state.

The binding of proflavin to pd(A-IU) produced a noticeable change in the low temperature total emission spectrum. The large phosphorescence band centered at 560 nm was of comparable intensity to the fluorescence band and is a result of a heavy atom perturbation of the proflavin molecule by the iodouracil. The enhancement of the phosphorescence was accompanied by a decrease in the fluorescence efficiency (Table I). The delayed fluorescence to prompt fluorescence ratio (Fig. 6) was greater than that of either the free or DNA-bound dyes (Figs. 4 and 5).

The enhancement of the delayed emission component of proflavin bound to pd(A-IU), which is readily apparent at low temperature, persists to room temperature. Under these room temperature conditions the total emission spectrum consisted of a single band centered at 505 nm of which 40% is delayed fluorescence. Phosphorescence at room temperature was negligible. In the other dye preparations the delayed fluorescence component of the room temperature total emission spectrum was small, <1%. The large enhancement in delayed fluorescence occurs as a result of the increase in intersystem crossing. Whereas this

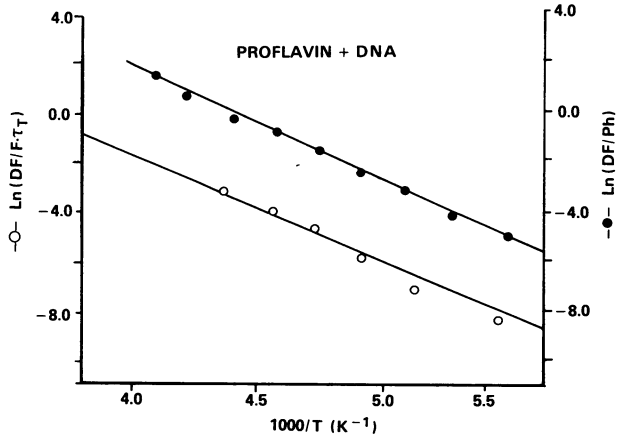


FIGURE 5 The variation of ln (DF/Ph) (closed data points) and ln (DF/F · $\tau_T$) (open data points) vs. reciprocal temperature for proflavin bound to DNA in glycerol/buffer solution (see caption of Fig. 4).

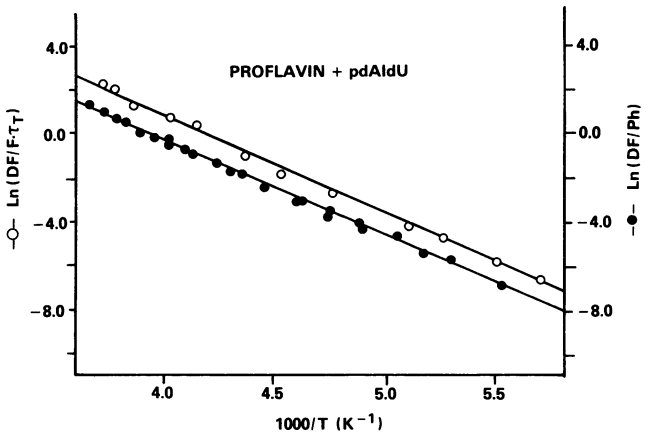


FIGURE 6 The variation of ln (DF/Ph) (closed data points) and ln (DF/F · $\tau_T$) (open data points) vs. reciprocal temperature for proflavin bound to pd(A-IU) in glycerol/buffer solution (see caption of Fig. 4).

results in the excitation being shunted to the triplet manifold with concomitant quenching of the prompt fluorescence, at sufficiently high temperatures the excitation is recycled back to the singlet state.

Parker and co-workers (Parker, 1964; Parker and Joyce, 1973) and Dixit et al. (1984) have presented Arrhenius expressions for the ratios of the delayed fluorescence to phosphorescence and delayed fluorescence to prompt fluorescence. These differ slightly from ours in that we have assumed a preequilibrium between the $T_1$ and T' states. A further assumption made by us was that $k_{ix} = k'_{ix}$. The latter is assumed in that the reverse intersystem crossing undoubtedly occurs from one of the three triplet sublevels which couples most strongly to the singlet state through spin-orbit coupling (Kwiram, 1967; Zulich et al., 1973). The values we present for the intersystem crossing could be regarded, however, as the harmonic mean of the foward and reverse processes.

## Analysis of Delayed Fluorescence and Phosphorescence of Proflavin

In the appendix we have derived expressions for the ratios of the delayed fluorescence to phosphorescence and the delayed fluorescence to prompt fluorescence, which are

$$\ln (DF/Ph) = \ln (k_f\phi_{ix}/k_p) - \Delta E/RT \quad (1)$$

$$\ln (DF/(F\tau_T)) = \ln (\phi_{ix}^2/\tau_S) - \Delta E/RT. \quad (2)$$

Arrenhius plots of Eqs. 1 and 2 predict slopes equal to $-\Delta E/R$ and Y-intercepts equal to $\ln (k_f\phi_{ix}/k_p)$ and $\ln (\phi_{ix}^2/\tau_S)$, respectively.

The Arrhenius plots of the DF/Ph and DF/(F · $\tau_T$) data for free, DNA-bound and pd(A-IU)-bound dye are shown in Figs. 4–6. The activation energies and *y*-intercepts obtained from these plots are summarized in Table II. The information contained in these figures in conjunction with the fluorescence efficiencies and the

TABLE II
ACTIVATION ENERGIES AND *y*-INTERCEPTS OBTAINED FROM THE ARRHENIUS PLOTS OF DF/Ph AND DF/(F · $\tau_T$) OF PROFLAVIN EMISSION

| Dye | DF/Ph | | DF/(F · $\tau_T$) | |
|---|---|---|---|---|
| | ΔE | *y*-intercept | ΔE | *y*-intercept |
| | *kJ mol*$^{-1}$ | | *kJ mol*$^{-1}$ | |
| Free | 37.0 | 19.0 | 36.8 | 13.8 |
| +DNA | 38.0 | 20.2 | 35.9 | 15.6 |
| +pdAIdU | 35.8 | 16.9 | 36.6 | 18.6 |

These data are shown in Figs. 4–6; ΔE was obtained from the slopes of the plots of Eqs. 8 and 14. All solutions were in 70/30 glycerol/buffer. +DNA and +pdAIdU see legend of Table I.

singlet and triplet lifetimes allows a complete description of the excited state processes in proflavin. A previous study by Parker and Joyce (1973) of the delayed and prompt fluorescence of free and DNA-bound proflavin yielded triplet-singlet energy splittings, although at higher temperatures than in the present work. Their analysis did not produce estimations of the intersystem crossing efficiency.

## Intersystem Crossing in Proflavin

We can use the data in Table II to obtain estimates of the intersystem crossing ($S_1 \rightarrow T_1$) in proflavin.

(*a*) *Free Proflavin.* For the free dye the *y*-intercept of equation 2 is

$$\ln (\phi_{ix}^2/\tau_S) = 13.84$$

or

$$\phi_{ix}^2/\tau_S = 1.03 \times 10^6.$$

With $\tau_s = 9.6 \times 10^{-9}$ s (see Appendix) we have $\phi_{ix} = 0.099$. From the *y*-intercept of Eq. 1,

$$\ln (k_f\phi_{ix}/k_p) = 19.0$$

or

$$k_p = k_f\phi_{ix}/(1.73 \times 10^8).$$

We can write $k_f = \phi_f/\tau_S$. Given that $\phi_f = 0.39$ (Parker and Joyce, 1973) and $\tau_S = 4.4 \times 10^{-9}$ s (Weill, 1965; Georghiou, 1975), then $k_f = 0.39/4.4 \times 10^{-9}$ s $= 8.9 \times 10^7$ s$^{-1}$. Substituting for $k_f$ and $\phi_{ix}$ above we have

$$k_p = (8.9 \times 10^7 \times 0.099)/(1.73 \times 10^8)$$
$$= 0.051 \text{ s}^{-1}.$$

Using these data and the triplet lifetime we can predict the ratio of phosphorescence to normal fluorescence at any particular temperature.

At −55°C the triplet lifetime of free proflavin in the sample was 0.60 s. Because $\phi_p = k_p\tau_T$, then $\phi_p = 3.1 \times 10^{-2}$. The ratio of phosphorescence to prompt fluorescence is Ph/F = $\phi_{ix}\phi_p/\phi_f$. At −55° $\phi_f = 0.85$ (Table I), and

therefore the predicted ratio of the phosphorescence to fluorescence intensities, Ph/F is equal to $0.099 \times 0.031/0.85 = 3.6 \times 10^{-3}$. This ratio can be compared with that of the corrected total emission spectrum (see Table I) at this temperature from which Ph/F was found to be equal to $8.5 \times 10^{-3}$.

(*b*) *DNA-bound Proflavin.* For proflavin bound to DNA at −55° we can make the same calculation as shown above. The singlet lifetime was taken to be $9.6 \times 10^{-9}$ (see Appendix). Using the *y*-intercept values given in Table II we obtained the following results.

$$\phi_{ix} = 0.23$$

$$k_p = 0.036\ s^{-1}.$$

At this temperature the triplet lifetime of DNA-bound proflavin was 0.25 s and $\phi_f$ was 0.76 (Table I), so that:

$$\phi_p = 0.036 \times 0.25 = 9.0 \times 10^{-3}$$

The ratio $Ph/F = 0.23 \times 0.0090/0.76 = 2.7 \times 10^{-3}$. From the corrected total emission spectrum at this temperature (Table I) $Ph/F = 3.1 \times 10^{-3}$.

(*c*) *pd(A-IU)-bound Proflavin.* At −55°C the singlet lifetime of proflavin bound to pd(A-IU) was taken to be $2.8 \times 10^{-9}$ s. The value of $\phi_f$ was 0.25. Using the data from Table II we obtained

$$\phi_{ix} = 0.58$$

$$k_p = 2.4\ s^{-1}.$$

At −55°C the triplet lifetime was 0.11 s, and therefore

$$\phi_p = 2.4 \times 0.11 = 0.26.$$

The predicted Ph/F ratio using these constants was $0.58 \times 0.26/0.25 = 0.60$ compared with a value of 0.39 obtained from the total emission spectrum (Table I). The proximity of the dye to the iodine atom results in a substantial increase in $k_p$ and Ph/F over that obtained for either the free or DNA bound dye (see Table I).

Over the temperature range of the DF measurements (−70 to −20°C) the values of $k_f$, $k_p$, $k_{ix}$ and $\tau_S$ remained relatively constant since the Arrhenius plots (Figs. 4–6) did not display any appreciable deviations from linearity.

## Internal Conversion in Proflavin

We have determined the rate constants and efficiencies of the internal conversion process for proflavin. We assumed that the primary deactivation pathways of the excited singlet state of proflavin are fluorescence, intersystem crossing, and internal conversion, that is,

$$\phi_f + \phi_{ic} + \phi_{ix} = 1.0.$$

From Table I the value of $\phi_f$ for free dye at −100° is 0.90 and $\phi_{ix}$ was determined to be 0.099 thus $\phi_{ic}$ is small.

$$\phi_{ic} = 1.0 - (0.90 + 0.099) = 0.001.$$

Similar results are obtained with DNA- and pd(A-IU)-bound dye: at low temperature the sum of $\phi_f$ and $\phi_{ix}$ is nearly unity. In the case of DNA this would suggest that the quenching of the proflavin fluorescence by neighboring guanosine-cytosine base pairs (Kubota et al., 1978) results in an enhancement of the efficiency of intersystem crossing. In each dye preparation $k_f$ and $k_{ix}$ are relatively constant over a range of increasing temperature where the fluorescence efficiency has been observed to decrease. These results would indicate that the decrease in $\phi_f$ that accompanies increasing temperature is a result of an increase in the rate of internal conversion ($k_{ic}$). The temperature dependence and influence of solvent mobility upon the internal conversion process has been the subject of another paper (Lee and Galley, unpublished).

## DISCUSSION

Time-dependent triplet state spectroscopy has been shown to be useful in the determination of the binding dynamics of proflavin to DNA (Corin and Jovin, 1985; Geacintov et al., 1982) and rotational motions in DNA-dye complexes (Hogan et al., 1982). The present study illustrates the potential of temperature-dependent studies to characterize the excited state processes in a chromophore. Most spectroscopic work has been performed at either room temperature or 77K and data obtained under either of these conditions provide limited amounts of information. Parker (1964) and Parker and Joyce (1973) provided earlier demonstrations of the usefulness of temperature-dependent triplet state spectroscopy.

The relative sensitivity for detecting the triplet state of proflavin via delayed emission spectroscopy is temperature dependent. Except at temperatures below about −40°C direct emission, i.e., phosphorescence, is a less efficient process than delayed fluorescence. This is due to the fact that the radiative decay rate constant of the triplet state, $k_p$, is small compared with the rate constants of the pathway leading to delayed fluorescence: $k_v$, $k_{ix}$, $k_f$ and that the triplet state is readily quenched. The fraction of the triplet emission that occurs as delayed fluorescence is governed by a Boltzmann distribution over the vibrational levels in the $T_1$ electronic state. When the temperature is high enough a sufficient portion of the triplet population exists in the thermally excited states to allow delayed fluorescence to be the main radiative pathway.

The room temperature triplet emission of proflavin can be compared with the room temperature emission of proteins. For proteins with sufficiently immobilized tryptophan residues, the only long-lived room temperature emission observed is phosphorescence (Saviotti and Galley, 1974). This contrasting behavior of the room temperature emission of tryptophan compared with proflavin is primarily due to the fact that the energy difference between the triplet and singlet states in tryptophan is greater than in proflavin: 6,900 vs. 2,400 $cm^{-1}$.

In addition to ΔE other parameters, $\phi_{ix}$ and $k_p$ are

important in determining DF/Ph and DF/F ratios. This is apparent from a comparison of the Arrhenius plots in Figs. 4–6. The slopes in all of these are essentially the same; the variable features are contained in the $y$-intercepts. The increase in $\phi_{ix}$ upon binding to both DNA and pd(A-IU) results in an increase in the DF/F($\tau_T$) ratios because the $y$-intercept is given by ln ($\phi_{ix}^2/\tau_S$). For the DF/Ph ratios the $y$-intercept is determined by ln ($k_f\phi_{ix}/k_p$). Binding of proflavin to DNA increases $\phi_{ix}$, the other two parameters are relatively constant, and the DF/Ph ratio is seen to increase. The heavy atom effect which accompanies the binding of proflavin to pd(A-IU) produces not only an increase in $\phi_{ix}$ but an even greater increase in the phosphorescence rate constant which results in a decrease in the DF/Ph ratio.

Making some assumptions about the singlet lifetimes we have been able to extract semiquantitative values for the excited state parameters of proflavin and to observe how these processes vary with temperature. At low temperatures in the absence of diffusional motions the rate of internal conversion is low. The decrease observed in the fluorescence efficiency of free and DNA-bound proflavin with increasing temperature is chiefly a result of an increase in the internal conversion process, $k_{ic}$ (Lee and Galley, unpublished observation).

The observation of the increase in the ratio of DF/($F\tau_T$) upon binding to DNA was made by Parker and Joyce (1973) although their experiments were carried out at warmer temperatures (4–60°C) and the Arrhenius plots of the delayed fluorescence to prompt fluorescence ratios were not linear over the complete temperature range.

Increases in $\phi_{ix}$ are associated with enhanced spin-orbit coupling in the molecule. Marked enhancements in spin-orbit coupling such as those induced by heavy atoms perturb the radiative and nonradiative pathways for the return of the molecule from the triplet level back to the ground singlet state as well as the $S_1 \rightarrow T_1$ intersystem crossing. However, there is no change in the triplet lifetime of proflavin upon binding to DNA. At 77K the phosphorescence decay of DNA-bound proflavin is a single exponential with lifetime equal to that of the free dye, 2.4 s. The selective increase in $S_1 \rightarrow T_1$ intersystem crossing is of a more subtle type than that observed with the halogenated polynucleotide. Intersystem crossing rates are dependent upon the energy difference between the two states involved, smaller energy differences resulting in enhanced radiationless transitions between states (Siebrand, 1967). From neither the spectra themselves nor the Arrhenius plots do we see any significant change in the $\Delta E_{T\text{-}S}$, so that this does appear to be a factor. It has been proposed that distortions of aromatic molecules away from planarity enhance the intersystem crossing process by increasing the mixing of the $S_1$ and $T_1$ states (El Sayed et al., 1973). Perturbations of this kind to the geometry of the intercalated proflavin molecule in its binding site are likely the basis for the observed enhancements. This may be the particular case for intercalation adjacent to G-C base pairs in that it is binding at these sites which is responsible for the fluorescence quenching (Kubota et al., 1978). It remains unclear at present why these subtle increases induced in spin-orbit coupling are not apparent in the triplet decay.

The binding of proflavin to pd(A-IU) causes a more dramatic change in the total emission spectrum (Fig. 2 *c*). The change in this case is clearly a result of heavy atom–induced spin-orbital coupling. As discussed above, for proflavin bound to pd(A-IU) the ratio of DF/Ph decreased whereas DF/($F\tau_T$) increased compared with those of the free dye. The heavy atom effect produces fluorescence quenching and an increase in the efficiency of triplet formation. The triplet state then recycles the delayed emission through the singlet state. This is reflected in the increase in $\phi_{ix}$ and a decrease in $\tau_S$ that appear in the expression for the $y$-intercept of Eq. 2.

The dramatic change that appears in the total emission spectrum of pd(A-IU)-bound dye at 77K persists to room temperature in deaerated samples in which close to half the emission is delayed fluorescence. This observation could be useful in providing a complimentary approach in the fluorescence microscopy of chromosomes to that introduced by Latt (Latt, 1973; Latt et al., 1985). Small regions involved in replication or repair into which halogen-containing nucleotides are incorporated, under observations of delayed emission, should emit above a dark background rather than as a fluorescence quenching.

In this paper we have presented several advances in triplet state spectroscopy. We have examined how factors such as the triplet-singlet energy gap, the intersystem crossing, and the temperature influence the emission. A temperature range through which both the delayed fluorescence and phosphorescence appear with appreciable and measurable intensities has been established. We have made use of this finding, discussed in another paper, whereby the depolarization of these emissions was employed to monitor anisotropic motions of DNA (Lee and Galley, 1984). The analyses of the delayed emission have allowed a complete description of the excited state processes over a temperature range from 77K to room temperature. This work has also presented a method directed toward the determination of intersystem crossing efficiencies.

## APPENDIX

## Derivation of Expressions for the Ratios of Emission Intensities

Fig. 1 provides a schematic description of the transitions between the electronic states of a chromophore molecule. The phosphorescence intensity, Ph, can be written as

$$Ph = k_p[T_1], \tag{A1}$$

where $k_p$ is the phosphorescence rate constant and $[T_1]$ is the concentration of chromophore molecules in the excited state $T_1$. T′ represents a

vibrationally excited level in the triplet manifold which is isoenergetic to the lowest excited singlet state $S_1$. We have assumed that the rates of intersystem crossing in the forward and reverse directions, $k_{ix}$ and $k'_{ix}$ are equal. Since $k_v$ and $k'_v$ are of the order of $10^{12}$ $s^{-1}$ and are much greater than $k_{ix}$ ($\sim 10^8$ $s^{-1}$) a preequilibrium exists between $T_1$ and T'. We can write

$$[T']/[T_1] = e^{-\Delta E/RT} \quad (2)$$

where $\Delta E$ is the energy difference between the lowest singlet and triplet excited states.

$$\Delta E = E(T') - E(T_1) = E(S_1) - E(T_1).$$

If we denote the excited singlet population that arises from intersystem crossing back from the triplet to the singlet manifold as $[S_1^*]$, then

$$DF = k_f[S_1^*]. \quad (3)$$

Using the steady-state approximation we can write

$$d[S_1^*]/dt = k'_{ix}[T'] - (k_f + k_{ix} + k_{ic})[S_1^*] = 0 \quad (4)$$

and

$$[S_1^*] = k'_{ix}\tau_s[T'] = \phi_{ix}[T']. \quad (5)$$

Substituting Eqs. 5 and 2 into Eq. 3,

$$DF = k_f\phi_{ix}[T_1]e^{-\Delta E/RT}. \quad (6)$$

Dividing Eq. 6 by Eq. 1 yields

$$DF/Ph = (k_f\phi_{ix}/k_p)e^{-\Delta E/RT} \quad (7)$$

or

$$\ln (DF/Ph) = \ln (k_f\phi_{ix}/k_p) - \Delta E/RT. \quad (8)$$

In Eq. 7 if $\Delta E \gg RT$, which will be shown to be so, then essentially all of the molecules in the triplet manifold are in the $T_1$ state. We can write

$$d[T_1]/dt = k_{ix}[S_1] - (k_p + k_{nr})[T_1] = 0$$

$$[T_1] = k_{ix}\tau_T[S_1], \quad (9)$$

where $\tau_T$ is the triplet lifetime. Substitution of Eq. 9 into Eq. 6 gives

$$DF = k_f k_{ix}\phi_{ix}\tau_T[S_1]e^{-\Delta E/RT}. \quad (10)$$

The prompt fluorescence intensity, F, can be written as:

$$F = k_f[S_1]. \quad (11)$$

Division of Eq. 10 by Eq. 11 yields

$$DF/F = \phi_{ix}k_{ix}\tau_T e^{-\Delta E/RT} \quad (12)$$

or

$$DF/(F\tau_T) = (\phi_{ix}^2/\tau_S)e^{-\Delta E/RT} \quad (13)$$

and finally

$$\ln [DF/(F\tau_T)] = \ln (\phi_{ix}^2/\tau_S) - \Delta E/RT. \quad (14)$$

The measurement of the prompt and delayed emission was conducted over a temperature range of 180 to 270K. At the warm end of this range the product of RT is equal to 2.3 kJ $mol^{-1}$, which is small compared with the values of $\Delta E_{T\text{-}S}$ ($\sim 36$ kJ $mol^{-1}$) given in Table II.

## Estimation of Singlet Lifetimes

The apparatus used in this work did not allow us to measure the singlet lifetimes of proflavin used in the kinetic analysis of the data. Instead we estimated these values from the known values of the singlet lifetime, 4.4 ns (Weill, 1965; Georghiou, 1975) and the fluorescence efficiency, 0.39 (Parker and Joyce, 1973) at room temperature. At temperature $t$ the singlet lifetimes can be expressed as:

$$\tau_{S,T} = (\phi_{f,t}/\phi_{f,22°C}) \times \tau_{S,22°C}.$$

At $-55$°C $\phi_f$ of the free dye was 0.85 (Table II) and

$$\tau_S = (0.85/0.39) \times 4.4 \text{ ns} = 9.6 \text{ ns}.$$

The singlet lifetime of proflavin bound to pd(A-IU) was determined in the same manner, $\phi_f$ at $-55$°C = 0.25,

$$\tau_S = (0.25/0.39) \times 4.4 \text{ ns} = 2.8 \text{ ns}.$$

For proflavin bound to DNA we assumed the singlet lifetimes to be equal to those of the free dye. Previous work has demonstrated that binding of proflavin to DNA did not alter the singlet lifetime appreciably even though the fluorescence efficiency was decreased. Weill (1965) found a singlet lifetime of DNA-bound proflavin to be equal to that of free proflavin whereas Georghiou (1975) observed only a slight increase in the lifetime of the bound form.

We can compare the value of $k_f$ determined from experimental data with that calculated from the absorption spectrum by the method of Strickland and Berg (1962). From experimental data we obtain

$$1/k_f = \tau_S/\phi_f = 4.4 \text{ ns}/0.39$$

$$= 11.3 \text{ ns}.$$

The calculated value is based on the oscillator strength of the proflavin molecule of ~9 ns. From this we can infer that the estimates of the singlet lifetimes are correct to within ~20%.

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