March, 1979] BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN, VOL. 52 (3), 693—696 (1979)

Delayed Excimer Fluorescence of Acridine Orange Bound to DNA

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(Received August 23, 1978)

The temperature dependence of long-lived emissions of Acridine Orange bound to DNA has been investigated. Only phosphorescence with a maximum close to 620 nm is observed in the range 77—*ca.* 150 K. With a rise in temperature, the delayed thermal fluorescence around 530 nm is observed in addition to phosphorescence at a high ratio of DNA phosphate to dye (*P*/*D*). With a decrease in *P*/*D* value, the delayed thermal fluorescence is replaced by a new delayed emission band around 640 nm. Its maximum wavelength is very close to that of dimer fluorescence of Acridine Orange in water. It has been shown that the emission results from two processes: (1) thermal excitation of the triplet state of the dimer to the excimer state and (2) excimer formation after the thermal excitation of the triplet state of the monomer to the first excited singlet state.

The binding of Acridine Orange (AO) to DNA has been extensively studied because of its biological effects.[1,2] Like many acridine derivatives, AO binds to DNA to form two types of complexes; one (Complex I) results from external binding where electrostatic forces play an important role,[3,4] while the other (Complex II) occurs at high DNA phosphate/dye (*P*/*D*) ratios, generally considered as an intercalation process.[5,6] As compared to other related dyes, the binding of AO presents a particularly complex aspect due, in part, to its high tendency to dimerize.[7,8] It is well known that fluorescence spectra of AO in aqueous solutions at room temperature strongly depend on its concentration.[9] With increasing concentration of the dye, its green fluorescence with a maximum near 540 nm is replaced by a red fluorescence with a maximum close to 650 nm.[9] AO, especially when bound to single-strand polynucleotides or denatured DNA at a low *P*/*D* value, also shows red fluorescence.[10–13] Such emission properties of the dye have been applied to studies of the conformational change of DNA and the nearest neighbor dye-dye interactions.[10–13]

In order to understand biological action of AO, it is of significance to clarify the nature of its excited state and the character of the interaction of the dye with the binding site, as well as the nearest neighbor dye-dye interactions. In previous papers,[14,15] reports were given on the phosphorescence, the delayed fluorescence due to triplet-triplet annihilation and the sensitized delayed fluorescence in the DNA–acridine dye complexes at 77 K. A study at low temperatures is of particular interest, since the radiationless processes which compete with the radiative processes (fluorescence and phosphorescence) are largely suppressed. Further, it is of interest to investigate the temperature dependence of the emission properties in order to elucidate the excited states of the bound dye.

The present paper describes the emission properties of AO bound to DNA over a wide temperature range and the newly-observed delayed emission.

Experimental

AO (Chroma) was purified by the method of Zanker.[9] No trace of impurity was detected by thin-layer chromatography on silica gel. Calf thymus DNA was obtained from Worthington Biochemical Corporation. The concentration of DNA was determined spectrophotometrically at 260 nm with the extinction coefficient per mole of DNA phosphate ($\varepsilon_p=6600\ \mathrm{M^{-1}\ cm^{-1}}$).[16] The thermal denaturation of DNA was performed by heating the DNA solution for 20 min in boiling water and then rapidly cooling in ice water. The solutions of the complexes were made up in 5 mM phosphate buffer at pH 6.9.

All the solutions were degassed by freezing, pumping, and thawing under a high vacuum. The solutions were placed in a quartz tube (inner diam. 1 mm) mounted in a Dewar flask with provision for the excitation and detection of the delayed emissions at right angles. For studies at 77 K the sample tube was immersed in liquid nitrogen. For measurements at various temperatures the temperature was controlled by a constant flow of cold nitrogen gas through the Dewar. The temperature was monitored by a copper-constantan thermocouple attached to the outside of the sample tube. The precision of the quoted values is ±1°.

The emission and excitation spectra were measured with a Hitachi MPF-2A fluorescence spectrophotometer; the detector was a red-sensitive photomultiplier tube, R446-UR (Hamamatsu Television Co.). Both spectra are given with corrections on the quantum response of detecting and exciting systems. A rotating-shutter phosphoroscope was used to separate the delayed emissions from normal fluorescence. The intensity of the exciting light was varied by introducing calibrated wire screens.

The absorption spectra at 77 K and at room temperature were measured with a Shimadzu MPS spectrophotometer with a 0.1 cm quartz cell.

Results and Discussion

Absorption Spectra of the Complexes. First, absorption spectra of the complexes were measured at room temperature and at 77 K in order to confirm the binding states of AO. It was found that the bound dye molecules predominantly exist as the monomeric species at a sufficiently high *P*/*D* value, tending to associate with a decrease in the *P*/*D* value. The association tendency is more remarkable in the denatured DNA and at 77 K than in the native one and at room temperature. Typical absorption spectra at 77 K are shown in Fig. 1. They exhibit a red shift of about 5 nm, being well resolved as compared with those at room temperature.[7,8,17] The 0-1 band around 470 nm in the monomer absorption spectrum generally overlaps the dimer absorption band.[7,9,18] As the *P*/*D* value decreases ($P/D<50$), the band around 470 nm becomes marked, while the 0-0 band around 500 nm is suppressed. This is ascribed to the formation of dye dimers. In the case $P/D<20$, the absorption

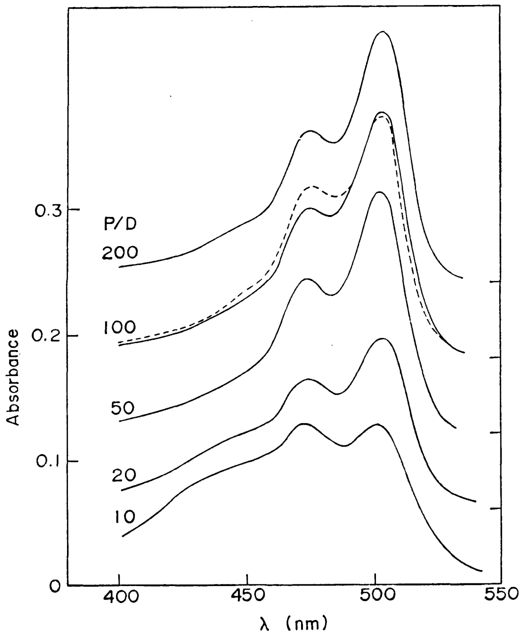

Fig. 1. Absorption spectra of DNA-AO complexes in 5 mM phosphate buffer at 77 K. The light-path length of the cell was 0.1 cm. For clarity of presentation the upper curves are displaced by a constant amount. Each line on the right ordinate corresponds to a base line. AO: 5.0×10^{-5} M, ——: native DNA, ----: denatured DNA.

band due to higher aggregates of the dye develops at wavelengths shorter than the 0-1 band around 470 nm.

Delayed Emission Spectra of the Complexes. Total emission and delayed emission spectra obtained under various temperatures are shown in Figs. 2A, 2B, and 2C. The spectral distribution of total emission shows little dependence on the temperature except that its maximum is slightly shifted to longer wavelength with increasing temperature. Total emission seems to be mainly composed of normal fluorescence, since its intensity is several ten times that of the delayed emission.

The delayed emission of the complexes consists only of phosphorescence with a maximum close to 620 nm in the range 77—*ca.* 150 K. With an increase in temperature, another delayed emission appears in addition to the phosphorescence; this phenomenon depends on the P/D value.

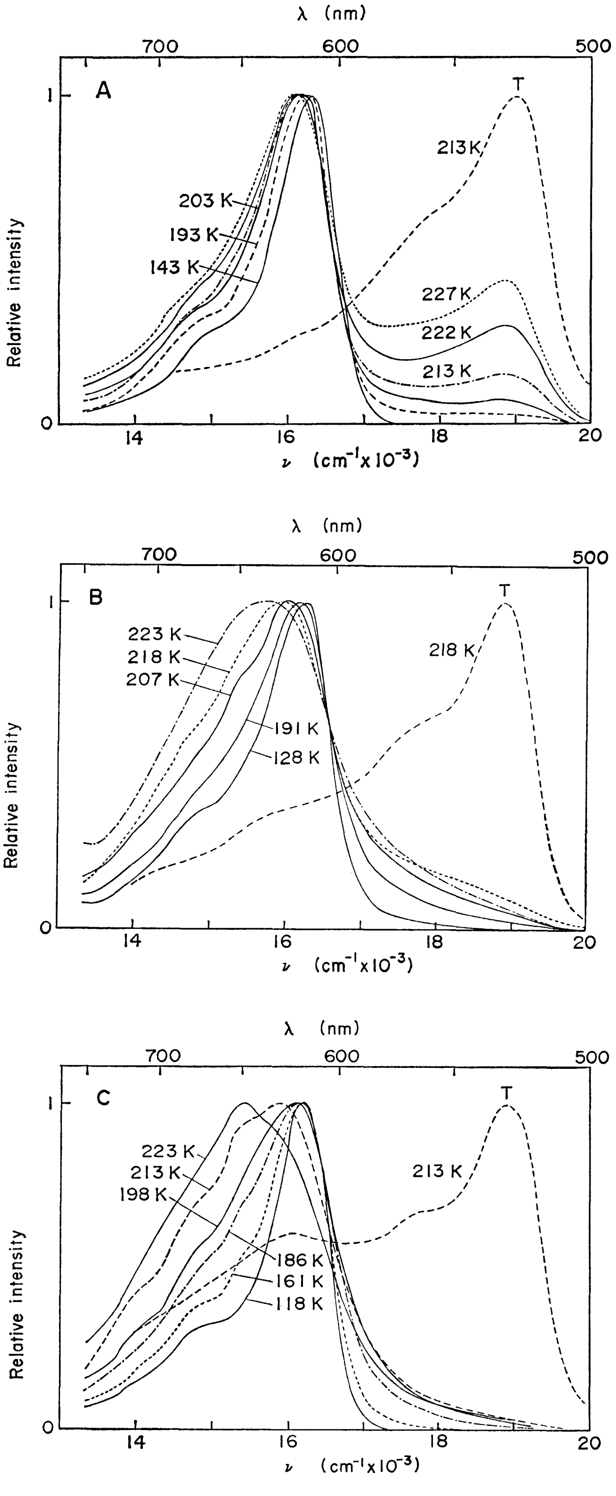

Fig. 2. (A) Normalized delayed emission spectra of DNA-AO complex ($P/D=302$) as a function of temperature. Excitation wavelength: 470 nm, AO: 5.0×10^{-5} M, T: total emission spectrum. (B) Same, $P/D=100$. (C) Same, $P/D=50$.

At a high P/D value ($P/D>100$), the delayed emission peaking at 530 nm becomes remarkable above *ca.* 200 K (Fig. 2A). The spectral distribution of delayed emission is very similar to that of normal fluorescence. The intensity of the delayed emission was found to be proportional to the first power of the intenisty of the exciting light, decaying exponentially with a lifetime equal to that of the phosphorescence. Thus, the delayed emission around 530 nm is due to the mechanism in which there is thermal activation from the lowest triplet state to the first excited singlet state followed by radiative transition from there to the ground state.[19,20] In the present study the delayed emission is called delayed thermal fluorescence according to the terminology of McGlynn *et al.*[20] The Arrhenius plot of ϕ_f/ϕ_p against $1/T$ were linear and the activation energy was obtained as 7.1 kcal mol^{-1}; here, ϕ_f and ϕ_p are the efficiencies of the delayed thermal fluorescence and phosphorescence, respectively. On the other hand, the peak-to-peak

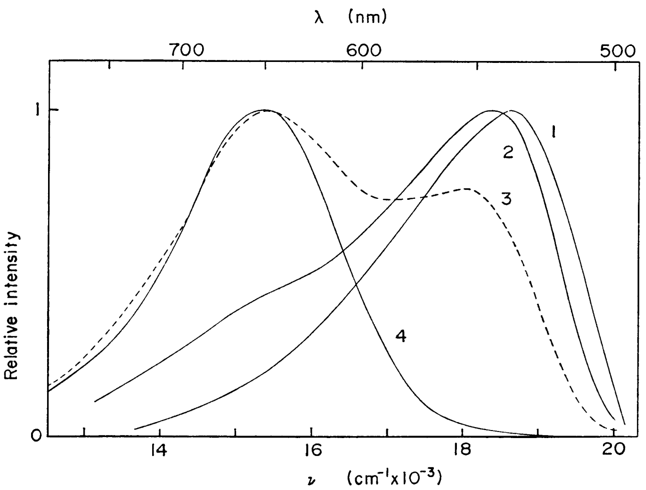

Fig. 3. Normalized fluorescence spectra of AO in 5 mM phosphate buffer at 25 °C. Excitation wavelength: 400 nm, AO: (1) 1.1×10^{-6} M, (2) 2.1×10^{-4} M, (3) 1.0×10^{-3} M, (4) 1.1×10^{-2} M.

separation between the delayed thermal fluorescence and the phosphorescence was 7.5 kcal mol^{-1}. Both values agree within experimental error, confirming the proposed mechanism.

With a decrease in P/D value ($P/D<120$), two interesting effects are observed (Figs. 2B and 2C): (1) a marked decrease in the intensity of the delayed thermal fluorescence as compared with the results at a high P/D value (Fig. 2A), and (2) appearance of a new emission band around 640 nm at elevated temperatures. AO easily associates to form a dimer or higher aggregates.[9] As shown in Fig. 3, fluorescence spectra of AO in aqueous solution highly depend on its concentration. With an increase in the concentration of dye, the green fluorescence with a maximum near 540 nm is replaced by a red fluorescence with a maximum close to 650 nm. The former is due to the dye monomer and the latter to the dye dimer or higher aggregates.[9] It should be noted that the shape of the delayed emission spectra peaking around 640 nm is very similar to that of a red fluorescence of the dye itself (Figs. 2B, 2C, and 3).

Excitation Spectra of the Delayed Emissions. Figure 4 shows typical results obtained with $P/D=50$. The excitation spectra of both the phosphorescence at 77 K and the delayed thermal fluorescence at 213 K ($\lambda_{\max}=$ 530 nm) are almost the same as the absorption spectrum at 77 K. On the other hand, the excitation spectrum of the delayed emission observed at 640 nm and at 213 K differs from that of the delayed thermal fluorescence at 530 nm. The excitation spectrum of the delayed emission at 640 nm shows an increase of the band at 470 nm, accompanied by the corresponding decrease of the band at 500 nm. This shape of the excitation spectrum is similar to that of the absorption spectrum at a low P/D value (Fig. 1).

Delayed emission around 640 nm was observed more remarkably in the case of denatured DNA and at lower P/D values where the absorption band due to the dye dimer becomes pronounced (Fig. 1).

Mechanism of the Newly-observed Delayed Emission.

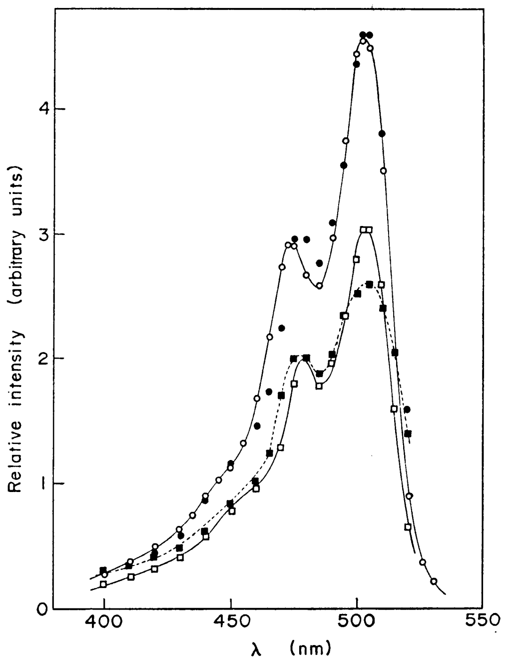

Fig. 4. Excitation spectra of delayed emissions of DNA-AO complex ($P/D=50$). ○: Absorption spectrum at 77 K, ●: excitation spectrum of phosphorescence observed at 610 nm and at 77 K, □: excitation spectrum of delayed thermal fluorescence observed at 530 nm and at 213 K, ■ : excitation spectrum of delayed emission observed at 640 nm and at 213 K.

A comparison of absorption, total emission and excitation spectra of the delayed emissions suggests that the dye dimer formed in the ground state may contribute to the newly-observed delayed emission. However, even at $P/D=100$ and $P/D=50$ where the monomeric form of the dye predominates, there is an intense delayed emission around 640 nm (Figs. 2B and 2C). This indicates that the newly-observed delayed emission can not be explained only by ground-state dimerization of the dye. An alternative explanation is that the newly-observed band is due, in part, to another type of dimeric species, known as excimer.

In the present case, the intensity of the delayed emission around 640 nm varies not with the second power but with the first power of the intensity of the exciting light. Thus, it seems that the delayed emission observed anew in this study is not due to triplet-triplet annihilation.[19]

It has been shown that the dimerization of AO leads to a red shift of *ca.* 30 nm in the peak of phosphorescence.[21] If we compare the phopshorescence spectra at 77 K, the shoulder around 660 nm becomes manifest at a low P/D value and in the case of denatured DNA where ground-state dimerization becomes significant; this is due to the dimer phosphorescence.[21]

In view of these findings, a mechanism similar to that of delayed thermal fluorescence may provide a good explanation for the above results.[22,23] The delayed emission around 640 nm can be interpreted as a result of two processes: 1. The thermal excitation from the triplet state of the dimer (D^t) to the excimer state (E), followed by an emission. 2. The excimer formation, $M_1+M_0 \rightarrow E$, after the thermal excitation of the triplet state of the monomer (M^t) to the first excited singlet state (M_1), followed by an emission; here, M_0 is the monomer in the ground state. The delayed emission due to this mechanism is then called delayed thermal excimer fluorescence. Because of the very small energy gap between D^t and E, the delayed thermal excimer fluorescence according to process 1 is expected to show up at considerably lower temperatures than the delayed thermal fluorescence (Figs. 2A, 2B, and 2C).

Formation of the Excimer. The bound dye is not uniformly distributed in DNA, but has a tendency to form clusters.[12)] The freezing of the DNA–dye solutions may accelerate the formation of clusters.[12)] The dye molecules in clusters exist closely and thus seem to be responsible for the dimer formation both in the ground and excited states.

In general, the bound dye molecules are considered to be intercalated between adjacent DNA base pairs at a high *P*/*D* value. Two models for the intercalation process have been proposed: complete intercalation model[5,6)] and partial intercalation model in which partly intercalated dye molecule is a possible binding site for additional, non-intercalated dye molecule.[7)] In the case of the former, the closest distance between bound dye molecules could be 10.2 Å assuming the exclusion of adjacent intercalation sites,[7,24)] while the closest distance could be 3.4 Å in the case of the latter. The mutual orientation and extent of overlap of adjacent dye molecules might be very important for the formation of the excimer. Observation of triplet-singlet and triplet-triplet energy transfers between DNA bases and dyes at 77 K shows that the structure of complexes at low temperatures is very similar to that at room temperature.[25,26)] If we judge from the distance between two bound dye molecules, the partial intercalation model seems favorable for the formation of the excimer.

In conclusion, it is evident that the nearest neighbor dye-dye interactions produce the excimer state of the dye. Such interactions may play an important role in the biological actions of AO.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education.

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