BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 43 3121—3125 (1970)

Luminescence in DNA-Acridine Dye Complexes. I. Phosphorescence and Delayed Fluorescence Due to the Triplet-Triplet Annihilation of Acridine Dyes in the Complexes

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(Received June 15, 1970)

When DNA-acridine dye complexes were excited by a visible light which was not absorbed by DNA, phosphorescence and a delayed fluorescence of the dye were observed. It was found, by comparing the excitation spectra with the absorption spectra, that the monomeric dyes mainly contribute to the fluorescence and phosphorescence emissions. The delayed fluorescence was interpreted as a result of the triplet-triplet annihilation which arises from two triplet dye molecules placed very close together. The decrease in the delayed fluorescence on the denaturation of DNA indicates that the interaction between the two triplets is sensitive to the conformation of the macromolecule.

The interaction of acridine dyes with DNA has been extensively studied by various techniques in connection with the photodynamic and mutagenic actions of these dyes. At present, it is generally accepted that these dyes bind to DNA to form two types of complexes; one (Complex I) results from the dye-dye interaction on the outside of the DNA helix at low P/D[1] values,[2,3] and the other (Complex II) occurs at high P/D values, where the dye-nucleotide interaction is involved.[4] Lerman has proposed that, in Complex II, acridine dye molecules are intercalated in a sandwich-like way between adjacent base pairs in the double-stranded helix.[5] For the understanding of the above biological actions of acridine dyes, therefore, it seems very significant to make clear the mechanism of the interactions between the DNA bases and dye molecules and between the dye molecules in the complexes from the photophysical and photochemical points of view.

In a previous communication,[6] it was shown that the triplet states of acridine dyes play an important role in the photosensitized radical formation in the complexes. In order to obtain further information regarding the excited states of acridine dyes and the energy transfer in the DNA-acridine dye complexes, the luminescence of the complexes was studied in frozen aqueous solutions at 77°K. In this study, proflavine, acridine orange, and acriflavine were used. Recently, the present author reported his preliminary results on the luminescence in the complexes.[7,8] The present paper will describe in detail the luminescence properties of acridine dyes in the complexes upon excitation by visible light.

1) The ratio of the number of nucleotides to that of acridine dye molecules.

2) D. F. Bradley and M. K. Wolf, *Proc. Natl. Acad. Sci. U. S.*, **45**, 94 (1959).

3) A. L. Stone and D. F. Bradley, *J. Amer. Chem. Soc.*, **83**, 3627 (1961).

4) A. R. Peacocke and J. N. H. Skerrett, *Trans. Faraday Soc.*, **52**, 261 (1956).

5) L. S. Lerman, *J. Mol. Biol.*, **3**, 18 (1961); *Proc. Natl. Acad. Sci. U. S.*, **49**, 94 (1963).

6) Y. Kubota and M. Miura, This Bulletin, **40**, 2989 (1967).

7) Y. Kubota, Y. Fujisaki and M. Miura, *Kogyo Kagaku Zasshi*, **72**, 252 (1969).

8) Y. Kubota, Y. Fujisaki and M. Miura, This Bulletin, **42**, 853 (1969).

Experimental

Materials. The acridine orange (Tokyo Kasei) was purified by the method of Zanker.[9] The proflavine sulfate (G. R. of Tokyo Kasei) was precipitated as a free base from an aqueous solution by adding a dilute sodium hydroxide solution. The precipitate was recrystallized three times from an ethanol-water mixture. The acriflavine (Chroma) was purified by the method of Gailliot[10] and was recrystallized twice from methanol. To check the purity, these dyes were subjected to silica-gel thin-layer chromatography; the chromatogram showed only a single colored band for each dye.

Calf thymus DNA (Type I) was obtained from the Sigma Chemical Co. The concentration of the DNA phosphates was determined by analyzing the phosphorus by the method of Chen *et al.*[11] The atomic extinction coefficient per phosphorus was found to be 6700, which is characteristic of a native DNA.

The solutions of the complexes were made up in a 0.01M phosphate buffer at pH 7.0. Each DNA solution contained 0.01M sodium chloride to protect it from denaturation. The thermal denaturation of DNA was performed by heating the DNA solutions for 20 min in boiling water and by then rapidly cooling them in ice-water.

Measurements. All the solutions were degassed by the freezing, pumping, and thawing technique under a high vacuum. The glass of the sample solution was made at 77°K in a quartz tube (1.5—2 mm in internal diameter). The emission and excitation spectra at 77°K were measured with a Hitachi MPF-2A fluorescence spectrophotometer; the exciting light source was a 150-W xenon lamp, and the detector was a R-106 photomultiplier. A rotating-shutter phosphoroscope was used to separate the delayed emissions (phosphorescence and delayed fluorescence) from normal fluorescence. No correction of the emission spectra was made for the spectral sensitivity of the detector. The excitation spectra were corrected for the spectral-energy distribution of the exciting light. The intensity of the exciting light was varied by introducing calibrated wire screens. The decay of the delayed emission was generally measured at its maximum wavelength by using a Hitachi V-104 synchroscope.

The absorption spectra were measured with a Shimadzu MPS spectrophotometer at 77°K or at room temperature, using 0.1- and 0.2-cm quartz cells. This apparatus can detect a large fraction of the forward scattered light in addition to the transmitted light; thus, it gives an accurate absorption. We could obtain the reproducible absorption spectra by subtracting the apparent absorption of the reference solution from that of the sample solution, in spite of the development of cracks and translucency in the glass.

The ESR measurements of the lowest triplet state of the acridine dyes in the complexes were made using a JES-3BX ESR spectrometer in an ethylene glycol-water (1 : 1 by volume) mixture at 77°K.

9) V. Zanker, *Z. Physik. Chem.* (Leipzig), **199**, 225 (1952).

10) M. Gailliot, *Quarterly J. Pharm. Pharmacol.*, **7**, 63 (1934).

11) P. S. Chen, Jr., T. Y. Toribara and H. Warner, *Anal. Chem.*, **28**, 1756 (1956).

Results and Discussion

Absorption Spectra of the Complexes. First, the absorption spectra of the complexes were measured at room temperature and at 77°K in order to ascertain the binding states of acridine dyes. It was found that the dye molecules predominantly exist as the monomeric species at a sufficiently high P/D value, and that they tend to associate with a decrease in the P/D value. The associative tendency appears to increase in the order of acridine orange, proflavine, and acrifivine and is more remarkable in the denatured DNA than in the native one. Typical examples of the absorption spectra at 77°K are shown in Figs. 1 and 2. They exhibit a red shift of about 10 nm and are well resolved as compared with those at room temperature.[2,4,12] The absorption band corresponding to the 0—1 transition generally overlaps with the dimer absorp-

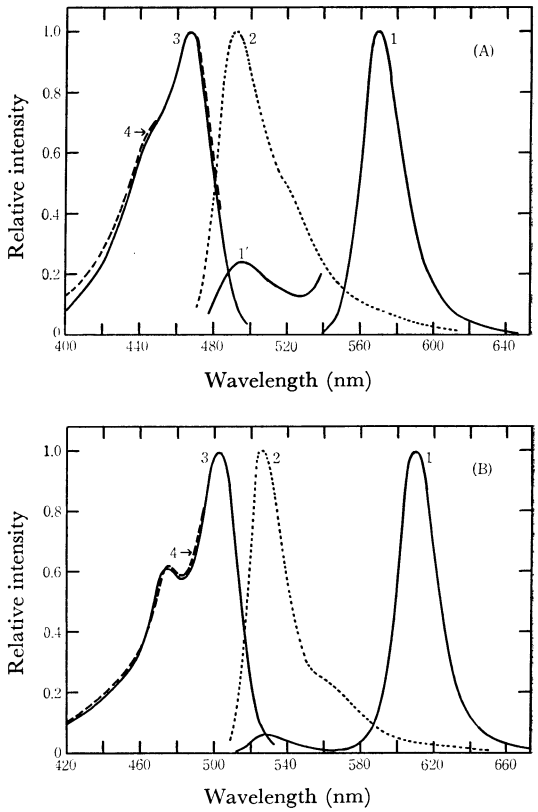

Fig. 1. The emission, excitation, and absorption spectra of (A) proflavine and (B) acridine orange in the complexes ($P/D=100$) at 77°K.
Excitation wavelength: (A) 460 nm, (B) 500 nm
Dye concn.: 5×10^{-5}M
(1) Long-lived emission, (1′) higher sensitivity of (1), (2) total emission, (3) absorption, and (4) phosphorescence-excitation spectra.

12) G. Weill and M. Calvin, *Biopolymers*, **1**, 401 (1963).

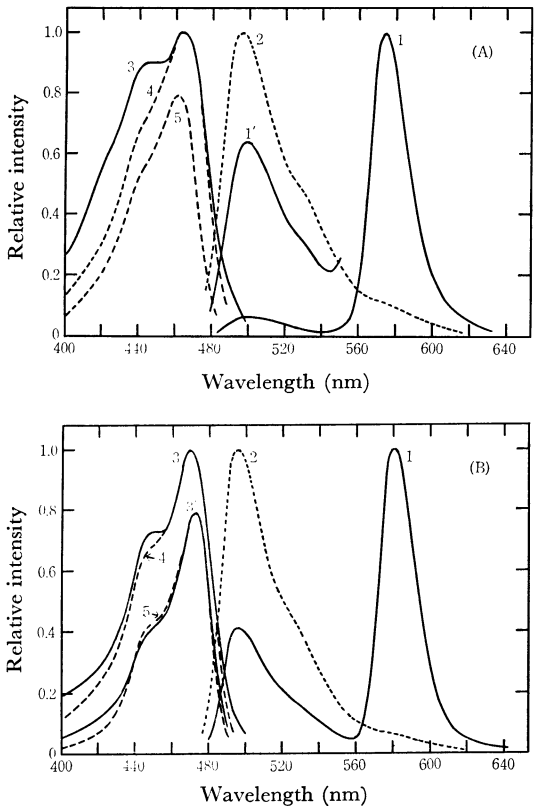

Fig. 2. The emission, excitation, and absorption spectra of (A) proflavine and (B) acriflavine in the complexes ($P/D=5$) at 77°K.
Excitation wavelength: (A) 460 nm, (B) 470 nm
Dye concn.: 5×10^{-5}M
(1) Long-lived emission, (1′) higher sensitivity of (1), (2) total emission, absorption spectra at (3) $P/D=5$ and (3′) $P/D=100$, and the excitation spectra of (4) phosphorescence and (5) delayed fluorescence.

tion band for each dye. In the case of $P/D<5$, absorption due to the aggregation of free dyes develops at wavelengths shorter than the 0—1 band.

Phosphorescence and Phosphorescence-Excitation (PE) Spectra of Acridine Dyes in the Complexes. The total emission and the delayed emission spectra of acridine dyes upon the excitation of the complexes by the visible light are shown in Figs. 1 and 2. The total emission seems to be mainly composed of normal fluorescence. The phosphorescence spectra at low dye concentrations (below 2.5×10^{-5}M) have peaks at 565, 604, and 574 nm for proflavine, acridine orange, and acriflavine respectively. These spectra are similar to those of acridine dyes in organic solvents at 77°K.[13,14]

With an increase in the dye concentration, both phosphorescence and fluorescence spectra slightly shift to longer wavelengths; this may be due to self-absorption. The phosphorescence intensity decreases with a decrease in the P/D value; this phenomenon becomes remarkable in the case of denatured DNA.

Next, the PE spectra were measured in order to obtain information on the phosphorescing species by comparing them with the absorption spectra. As Figs. 1 and 2 show, the PE spectra at high and low P/D values are approximately identical and agree well with the absorption spectra at a high P/D value. Further, it was found that the PE spectra are identical with the fluorescence-excitation spectra. These results indicate that the monomeric dyes mainly contribute to the fluorescence and phosphorescence emissions. Thus, the decrease in the phosphorescence intensity with a decrease in the P/D value or in the case of denatured DNA may be interpreted as a result of the increase in the number of dimeric species.

Table 1. Phosphorescence lifetimes of acridine dyes in the complexes at 77°K (sec)

Dye concn. (M)	P/D	Acridine orange (500 nm)*1	Proflavine (460 nm)*1	Acriflavine (470 nm)*1
10^{-4}	100	1.27	1.77	1.89
5×10^{-5}	100	1.34	1.90	1.96
		1.34*2		1.95*2
	50	1.31	1.88	1.94
		1.31*2		1.94*2
	10	1.25*3	1.81*3	1.90*3
	5		1.76*3	1.86*3
2.5×10^{-5}	200	1.50	1.98	2.06
10^{-5}	500	1.60	2.00	2.08

*1 Excitation wavelength.
*2 Denatured DNA.
*3 Slightly deviates from the exponentiality at the initial stage.

Finally, the mean lifetimes of the phosphorescence are summarized in Table 1. In general, these values are a little smaller than those in organic solvents at 77°K (*cf.* Table 2).[13]

ESR of the Lowest Triplet State of Acridine Dyes in the Complexes. The ESR spectra corresponding to the $\Delta M=2$ transitions of acridine dyes in the complexes were observed at a magnetic field of *ca.* 1600 gauss. From the maximum position on the low-field side of the derivative spectrum, one can calculate the root-mean-square zero-field splitting parameter (D^*), which is a measure of the electron spin-spin interaction.[15] As may be seen from Table 2, the D^* value at a high P/D

13) The preliminary results have already been presented; Y. Kubota and M. Miura, This Bulletin, **42**, 2763 (1969).

14) R. W. Chambers and D. R. Kears, *J. Phys. Chem.*, **72**, 4718 (1968); *Photochem. Photobiol.*, **10**, 215 (1969).

15) J. H. van der Waals and M. S. de Groot, *Mol. Phys.*, **2**, 333 (1959); *ibid.*, **3**, 190 (1960).

TABLE 2. ESR RESULTS OF THE TRIPLET STATE OF ACRIDINE DYES IN THE COMPLEXES AT 77°K

DNA (M in phosphate unit)	Acridine orange		Proflavine	
	D^* (cm^{-1})	τ (sec)	D^* (cm^{-1})	τ (sec)
0	0.0714	1.8	0.0795	2.2
1.25×10^{-2}	0.0726	1.9		
2.5×10^{-2}	0.0728	1.9	0.0805	2.2

τ: lifetime of the triplet state
Dye concn.: 5×10^{-4}M
Solvent: ethylene glycol-phosphate buffer (1 : 1 by volume, pH 7.0) mixture

value is in good agreement with those corresponding to the monomeric dyes in organic solvents.[13] Therefore, the ESR results also support the idea that the monomeric dyes mainly contribute to the phosphorescence emission.

Delayed Fluorescence Due to the Triplet-Triplet Annihilation of Acridine Dyes in the Complexes. As Figs. 1 and 2 show, a delayed fluorescence of the dye was also observed. The peak of the delayed fluorescence shifts to a slightly longer wavelength, but this is trivial; this may be attributed to self-absorption. This delayed fluorescence has not yet been reported in the DNA-acridine dye complexes; an effort will be made hereinafter to investigate its nature.

The excitation spectrum of the delayed fluorescence coincides well with the absorption spectra at a high P/D value, where most of the dye molecules exist as the monomeric species (see Fig. 2). Therefore, it may be concluded that the excitation process of the delayed fluorescence is identical with that of the fluorescence or phosphorescence of the monomeric dyes.

Since the thermal excitation from the lowest triplet to the first excited singlet state is actually impossible at 77°K for acridine dyes, the delayed fluorescence observed cannot be interpreted as an α-phosphorescence; this phenomenon was found to become remarkable at temperatures above 200°K.

Following the method of Azumi and McGlynn,[16] we plotted the intensity of the delayed fluorescence against the square of the phosphorescence intensity; this gave a straight line, as is shown in Fig. 3. Further, as is shown in Fig. 4, the growth of the delayed fluorescence was observed to depend on the initial triplet concentration and to be nearly equal to the growth of the triplet concentration after a long period of darkness.[17] In view of these results, it seems reasonable to conclude that two triplets are involved in the phenomenon of the delayed fluorescence—that is, two neighboring

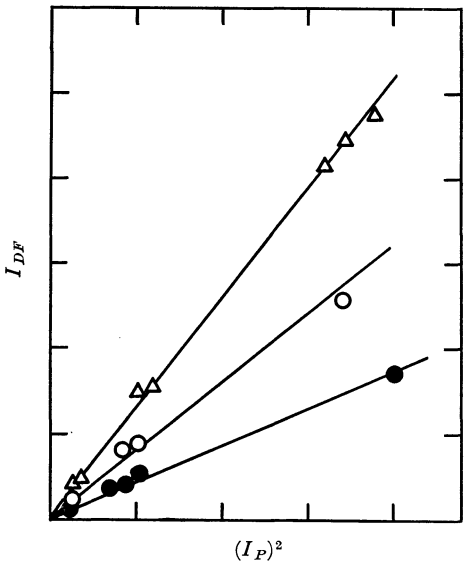

Fig. 3. A relationship between the intensity of delayed fluorescence (I_{DF}) and the square of the phosphorescence intensity (I_P).
△, acriflavine; ○, proflavine; ●, acridine orange

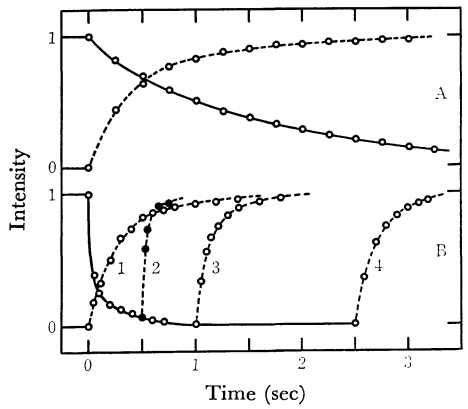

Fig. 4. The decay (solid line) and growth (dashed line) curves of (A) phosphorescence and (B) delayed fluorescence of acriflavine in the complexes at 77°K excited at 470 nm.
Acriflavine concn.: 5×10^{-5}M, P/D: 5
Period of darkness: (1) longer than 60 sec, (2) 0.5 sec, (3) 1 sec, and (4) 2.5 sec

triplet molecules produce one singlet molecule, which is then responsible for the delayed fluorescence. Moreover, the first period of the decay of phosphorescence shows deviations from the exponentiality (Fig. 5); these deviations may be related to the existence of the triplet-triplet annihilation process.[16,18]

16) T. Azumi and S. P. McGlynn, *J. Chem. Phys.*, **39**, 1186 (1963).
17) C. A. Parker, *Trans. Faraday Soc.*, **60**, 1998 (1964).
18) H. Sternlicht, G. C. Nieman and G. W. Robinson, *J. Chem. Phys.*, **38**, 1326 (1963); **39**, 1610 (1963).
19) *e. g.*, R. K. Tubbs, W. E. Ditmars, Jr., and Q. van Winkle, *J. Mol. Biol.*, **9**, 545 (1964).

It is well known that the probability of the triplet-triplet annihilation falls off rapidly with an increase in the triplet-state separation. As may be seen in Fig. 1, the delayed fluorescence can be observed even at P/D values higher than 100. If the dye binding is homogeneous, the interaction between two triplets is unlikely to occur at $P/D \geq 100$. Consequently, it may be thought that the binding is heterogeneous and that, thus, the assembly of dye molecules is partly formed in the double-stranded helix. This is consistent with the results previously obtained.[19]

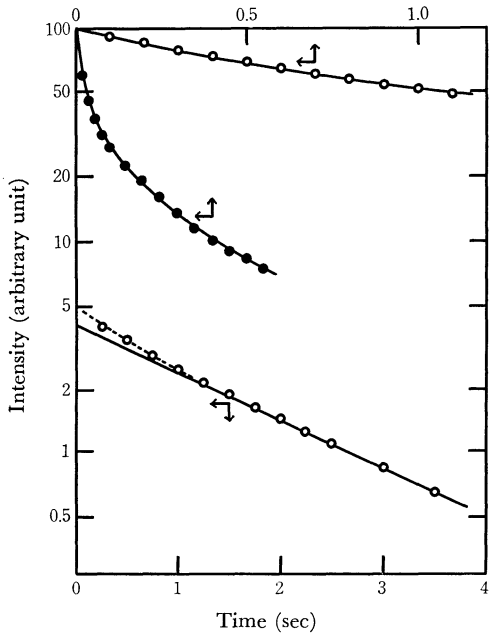

Fig. 5. The decay curves of phosphorescence (○) and delayed fluorescence (●) of acriflavine (5×10^{-5}M) in the complexes ($P/D=5$) at 77°K excited at 470 nm.
Phosphorescence decay in two parts: 0—1 sec and 1—4 sec.

On the other hand, if the triplet molecules are uniformly distributed and if the second-order processes are negligible, the delayed fluorescence will decay exponentially with twice the rate constant of phosphorescence decay.[16,18] In fact, as is shown in Fig. 5 in connection with the DNA-acriflavine complex, the decay of delayed fluorescence is not exponential; it decays very fast at the begining and then gradually becomes slower. The decay mode of the delayed fluorescence was independent of the P/D value and was similar to those of other acridine dyes. As has been mentioned above, a random distribution of dye molecules in the complexes is somewhat unlikely. Therefore, the initial rapid decay of the delayed fluorescence may be ascribed to the strong interaction between triplet molecules spaced very close together.

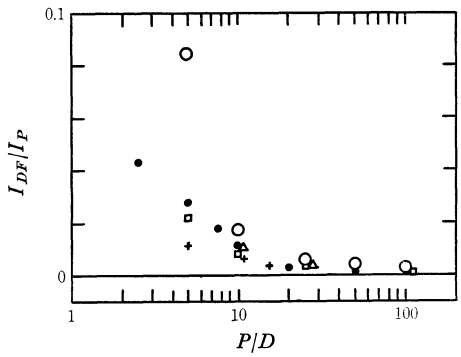

Fig. 6. The ratio of the intensity of delayed fluorescence to that of phosphorescence of the dye (I_{DF}/I_P) as a function of P/D. Both intensities are corrected for the spectral sensitivity of the detector.
○: acriflavine (5×10^{-5}M, I_{DF}/I_P values are 0.10 and 0.16 at $P/D=4$ and 2.5 respectively)
●: acriflavine (10^{-4}M)
+: acriflavine (10^{-4}M, denatured DNA)
□: proflavine (5×10^{-5}M)
△: acridine orange (5×10^{-5}M)

As may be seen in Fig. 6, the phenomenon of the delayed fluorescence seems to depend on the P/D value, the dye concentration, the kind of acridine dye, and the conformation of the macromolecular complex. First, the delayed fluorescence becomes remarkable with a decrease in the P/D value; this may be expected from the decrease in the mean distance between dye-dye molecules—that is, between two triplets. Second, the delayed fluorescence becomes weak with an increase in the dye concentration at a constant P/D value; this may be expected from the fact that dye association becomes more pronounced as the dye concentration increases. Third, the delayed fluorescence is observed most remarkably for acriflavine; this may be understood on the basis of the fact that acriflavine associates less than proflavine and acridine orange do. Finally, the denaturation of DNA leads to a marked diminution in the delayed fluorescence; this may be due to the difference between the conformations of the native and denatured DNA, where dye molecules associate remarkably. In the case of RNA, the delayed fluorescence is greatly decreased. Further, no triplet-triplet annihilation was found in the nucleotide-dye systems. Therefore, the double helical structure seems suitable for producing the annihilative process.

The author wishes to thank Professor Masaji Miura, Hiroshima University, for his helpful discussions and suggestions during this work. Thanks are also due to Professor Yasuo Fujisaki, Yamaguichi University, for his continuing interest and encouragement.