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The Excited States of Acridine Dyes. I. An ESR Study of the Triplet State

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The lowest triplet state of acridine dyes has been studied by an ESR technique over a wide range of concentration and in various solvents. The ESR spectra of the triplet state have been observed at a resonance field of *ca.* 1550 gauss, corresponding to the $\Delta M=2$ transitions. The ESR data characteristic of the triplet state depend on the dye concentration; this has been interpreted as being due to the association of dye molecules. The values of the zero-field splitting parameter, D^*, calculated from the $\Delta M=2$ transition spectra lead to the assignment of the lowest triplet state of acridine dyes as the $(\pi-\pi^*)$ state.

Since the first observation of the ESR spectra of the phosphorescent triplet state of naphthalene molecules in a single crystalline matrix[1)] and the ESR observations of the triplet state of randomly-oriented molecules in rigid glass solutions for the forbidden ($\Delta M=2$) transitions[2)] and for the allowed ($\Delta M=1$) transitions,[3,4)] the ESR technique has provided a powerful tool for the investigation of the triplet states of aromatic, heteroaromatic, and biological molecules. From the ESR measurements, information regarding the zero-field splitting (ZFS) parameters, D and E, the lifetime of the triplet state, and so on may be obtained.

Acridine dyes are of biological interest with regard to their photodynamic and mutagenic actions. It is of great significance to ascertain the nature of the excited states of acridine dyes, since their excited states, especially triplet states, are considered to play an important role in the primary process of photodynamic action.[5)]

For this paper, the lowest triplet state of several acridine dyes, mainly acridine orange, proflavine, and acriflavine, have been investigated by the ESR technique over a wide range of concentration and in various solvents.

Experimental

Materials. The acridine orange (Tokyo Kasei) was precipitated as a free base from the aqueous solution by adding a dilute sodium hydroxide solution. The resulting precipitate was dissolved in chloroform, and then the solution was passed through an activated alumina column in chloroform.[6)] The eluate containing a main band was filtered to remove any alumina particles. The free base was precipitated by evaporating chloroform under a reduced pressure and was then recrystallized three times from an ethanol-water mixture.

The proflavine (Tokyo Kasei) was also precipitated as a free base in a similar manner. The resulting free base was recrystallized twice from an ethanol-water mixture.

The acriflavine (Tokyo Kasei) was treated twice with freshly-precipitated silver hydroxide in order to remove the proflavine[7)] and was then recrystallized twice from methanol.

The acridine (Tokyo Kasei) was recrystallized three times from ethanol. The acridine yellow and acridine red (Tokyo Kasei) were used without further purification. The anthracene (Tokyo Kasei) was recrystallized from ethanol, and the naphthalene (Wako Junyaku) was purified by the sublimation method.

The methanol, ethanol, ether, and ethylene glycol (EG; G. R. of Wako) were purified according to the usual methods. The glycerine (G. R. of Wako) was used without further purification.

Apparatus and Procedures. The ESR spectra were measured by using a JES-3BS-X spectrometer with a field modulation of 100 kc/sec and with a TE_{011} mode cavity; the spectrometer had been manufactured by the Japan Electron Optics Laboratory. The microwave frequency was 9100—9200 Mc/sec, and the amplitude of the modulation field was 12—20 gauss (G), peak to peak. The sample solution was kept in a quartz tube (3 mm in inner diameter) and was kept at 77°K in a quartz Dewar. Glucose glasses were made by rapid cooling after fusing an appropriate

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1) C. A. Hutchison, Jr., and B. Mangum, *J. Chem. Phys.*, **29**, 952 (1958); *ibid.*, **34**, 908 (1961).

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

3) W. A. Yager, E. Wasserman and R. M. R. Cramer, *J. Chem. Phys.*, **37**, 1148 (1962).

4) E. Wasserman, L. C. Snyder and W. A. Yager, *ibid.*, **41**, 1763 (1964).

5) Y. Kubota and M. Miura, to be published.

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

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

mixture of glucose and dye; they were then kept in a desiccator with phosphorus pentoxide. Measurements were carried out at 77°K and at higher temperatures. The temperature, except at 77°K, was controlled by varying the flow rate of cold nitrogen gas through the cavity. The light sources were as follows: (1) a 750-W tungsten projector lamp with a filter (1 cm thick) containing 10% $CuSO_4$ aqueous solution, and (2) a 1-kW high-pressure mercury lamp with a filter (5 cm thick) containing a 0.1 M $NiSO_4$ aqueous solution and with two quartz lenses. The latter was used for the excitation of acridine, naphthalene, and anthracene, and the former, for the excitation of the other substances.

The absorption spectra were measured at room temperature with a Shimadzu QV-50 spectrophotometer. The phosphorescence measurements were performed at 77°K with a Hitachi MPF-2A fluorescence spectrophotometer (R-136 photomultiplier) with an attachment for the phosphorescence measurements.

Results and Discussion

The ESR spectra of the triplet state of acridine dyes were observed at a resonance field of *ca.* 1550 G corresponding to the $\Delta M=2$ transitions, which are shown in Fig. 1 for acridine orange, proflavine, and acriflavine. According to van der Waals and de Groot,[2] the position of the peak on the low-field side of the derivative spectrum (H_{min}) is related to the root-mean-square ZFS parameter, D^*, by the following equation:

$$D^* = (D^2+3E^2)^{1/2} = \sqrt{3}\,[1/4(h\nu)^2-(g\beta H_{min})^2]^{1/2}$$

where h is Plank's constant; ν, the microwave frequency; g, the spectroscopic splitting factor, and β, the Bohr magneton. The ESR results are summarized in Tables 1 and 2, where ΔH denotes the linewidth determined as the peak-to-peak distance of the derivative spectrum and where τ is the decay time of the ESR signal after the light has been extinguished; τ should be equal to the measured lifetime of phosphorescence.

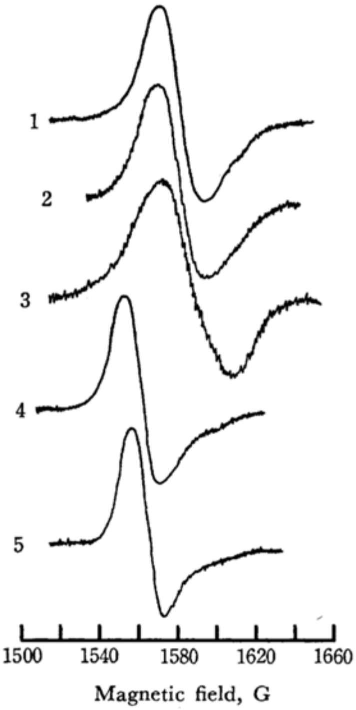

Fig. 1. ESR spectra of the triplet state of acridine dyes in glycerine at 77°K.
Microwave frequency: 9135±1 Mc/sec
(1) acridine orange: 9.7×10^{-5} M
(2) 1.3×10^{-3} M
(3) 6.5×10^{-3} M
(4) proflavine: 1.9×10^{-4} M
(5) acriflavine: 1.8×10^{-4} M

ZFS Parameters. Each D^* value for acridine dyes is constant within the range of experimental error, regardless of the dye concentration and of the kind of solvent at relatively low concentrations. At higher concentrations and in solvents containing water, however, the D^* value tends to decrease with an increase in the dye concentration. This will be discussed later. The D^* values given in Table 2 are the averages at low dye concentrations where the decrease of D^* value does not occur. These values are of the same order of magnitude as those of anthracene and naphthalene;[8] they may, therefore, make it possible to assign the lowest triplet state of acridine dyes to the (π-π^*) state. For acridine, its D^* value also suggests that the lowest triplet state is the (π-π^*) state.

In addition to the $\Delta M=2$ transition spectra, we could observe the $\Delta M=1$ transition spectra consisting of six lines associated with three molecular axes. The latter spectra were, however,

Table 1. The effect of concentration on ESR data of acridine dyes in glycerine at 77°K

Concn. (M)	D^* (cm^{-1})	ΔH (G)	τ (sec)
(A) Acridine orange			
9.7×10^{-5}	0.0724	25.0	1.8
1.7×10^{-4}	0.0732	25.2	
6.6×10^{-4}	0.0726	26.1	
1.3×10^{-3}	0.0723	26.5	
3.9×10^{-3}	0.0716	31.0	1.7
6.5×10^{-3}	0.0709	35.4	1.6
(B) Proflavine			
1.9×10^{-4}	0.0807	20.0	2.1
3.4×10^{-3}	0.0809	20.8	2.0
9.8×10^{-3}	0.0800	25.2	—
(C) Acriflavine			
1.8×10^{-4}	0.0786	19.0	2.2
2.2×10^{-3}	0.0790	19.5	2.2
8.1×10^{-3}	0.0783	24.8	2.1

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[8] B. Smaller, *J. Chem. Phys.*, **37**, 1578 (1962).

Table 2. Zero-field-splitting parameters of acridine dyes in various solvents at 77°K

Compound	Solvent	D^* (cm^{-1})	D (cm^{-1})	E (cm^{-1})	$\bar{\nu}_p$[d] (cm^{-1})
Acridine orange	Glycerine	0.0726	0.0636	0.0159	16500
	EG	0.0728			
	EG-water (1:1)[a]	0.0718			
	Methanol	0.0723			
	Methanol-water (1:1)[a]	0.0710			
	Ethanol	0.0723			
	Glucose[b]	0.0732			
Proflavine	Glycerine	0.0807	0.0734	0.0177	17500
	EG	0.0806			
	EG-water (1:1)[a]	0.0798			
	Methanol	0.0802			
	Glucose	0.0803			
Acriflavine	Glycerine	0.0788			17300
	Glucose[c]	0.0795			
Acridine	Ether	0.0688			
Acridine yellow	Glycerine	0.0725			
Acridine red	Glycerine	0.0705			
Naphthalene	Ethanol	0.1060			
Anthracene	Ethanol	0.0773			

a) Dye concn., 10^{-3} M.
b) Measured at 88°K.
c) Measured at 91°K.
d) The 0-0 band of phosphorescence.

relatively broader and much less intense than the former spectra because of their anisotropic character. From the $\Delta M=1$ transition spectra, the values of the ZFS parameters, D and E, were calculated;[3,4] they are presented in Table 2. The D^* values obtained from these D and E values, 0.0693 and 0.0795 for acridine orange and proflavine respectively, are in fairly good agreement with those obtained from the $\Delta M=2$ transition spectra.

In order to examine the temperature dependence of the D^* value, ESR measurements of glucose glass containing acridine dyes were carried out over the temperature range from 77 to 300°K. The intensities of both the phosphorescence and the ESR signal at the $\Delta M=2$ transition decrease with an increase in the temperature. The signal-to-noise ratio at 300°K was 4 : 1 for acridine orange. For proflavine and acriflavine, no ESR signals of the triplet state were observed at room temperature; instead, another strong green luminescence (probably α-phosphorescence) was observed for these dyes. As may be seen in Fig. 2 for acridine orange and acriflavine, it was found that the D^* values decrease with a rise in the temperature; namely, the plot of D^* against the temperature in each case gives an approximately straight line with a slope of -2×10^{-5} cm^{-1} deg^{-1}. This phenomenon, similar to that of coronene in a plastic matrix,[9] seems general in the triplet state of aromatic molecules.

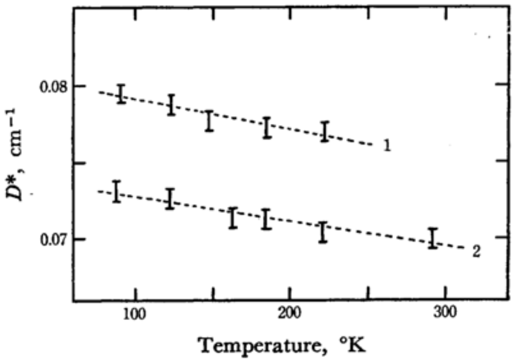

Fig. 2. Variation of D^* with temperature.
(1) Acriflavine, (2) Acridine orange

Lifetime of the Triplet State. As an example, the decay curve of the triplet state of proflavine is shown in Fig. 3 (a). The decay curve was generally exponential except for the first stage, up to 0.3 sec; this deviation may be due to the response time (0.3 sec) of the recorder. As is shown in Fig. 3(b), a plot of the logarithm of the signal intensity against the time gives a straight line; the lifetime of the triplet state can be calculated from the slope of this line. The lifetime, generally, tends to decrease with an increase in the dye concentration and is in good agreement with the results obtained from the phosphorescence measurements (see Fig. 5).

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9) C. Thomson, *ibid.*, **41**, 1 (1964).

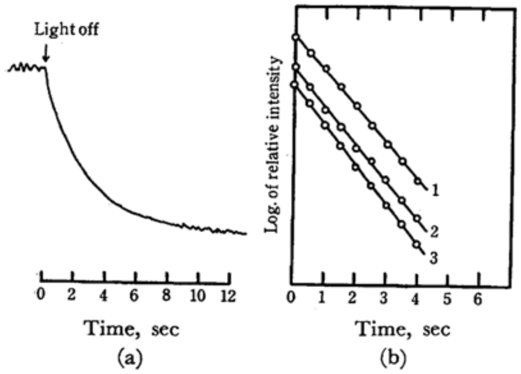

Fig. 3. Decay of the triplet state of acridine dyes in glycerine at 77°K.
(a) Proflavine: 1.9×10^{-4} M
(b) (1) Acriflvaine: 1.8×10^{-4} M
(2) Proflavine: 1.9×10^{-4} M
(3) Acridine orange: 1.7×10^{-4} M

Dependence of the ESR Data on the Dye Concentration. As may be seen from Table 1, the ESR data characteristic of the triplet state seem to depend on the dye concentration. Figure 4 shows the concentration dependence of the D^* and ΔH values in the case of acridine orange. In general, D^* decreases and ΔH increases with an increase in the dye concentration. This phenomenon, depending on the kind of solvent, was more remarkable in glycerine than in EG. As is well known,[6,10,11] acridine dyes easily form the dimer and higher aggregates at high concentrations in both water and organic solvents. First, the absorption spectra were measured at various concentrations in order to establish the associative properties of acridine dyes. These dyes were found, by the method of Zanker[10] and Mataga,[11] to exist in a monomer-dimer equilibrium in glycerine and EG. The equilibrium constants for the dimerization of acridine orange were estimated to be about 450, 100, and 460 M^{-1} in glycerine, EG, and EG-water (1 : 1 by volume) respectively. Further, the association tendency is considerably enhanced by a drop in the temperature.[10] Second, the ΔH value was unchanged irrespective of the triplet-state concentration, which was controlled by varying the intensity of the exciting light. This fact appears to rule out the possibility that the broadening of the linewidth arises from the interaction between triplet-state molecules, even though the intermolecular distance may be expected to be shortened at a high concentration. Next, the interaction between triplet-state molecules and radicals formed during irradiation may give rise to the broadening of the linewidth. The radical concentration was, however, too low to permit the identification of the radicals, and so this effect may be disregarded. Finally, the ESR data were independent of the concentration in the case of aromatic hydrocarbons which do not tend to associate, while they were concentration-dependent in the case of acridine dyes.

In view of these facts, it seems reasonable to assume that the association of acridine dyes is responsible for the apparent decrease in the D^* value and the apparent increase in the ΔH value

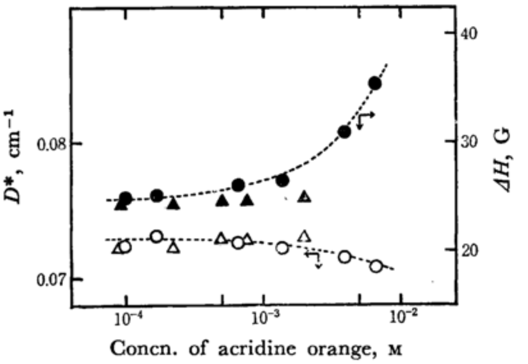

Fig. 4. Variation of D^* and ΔH with concentration of acridine orange at 77°K.

○ ●: in glycerine, △ ▲: in EG

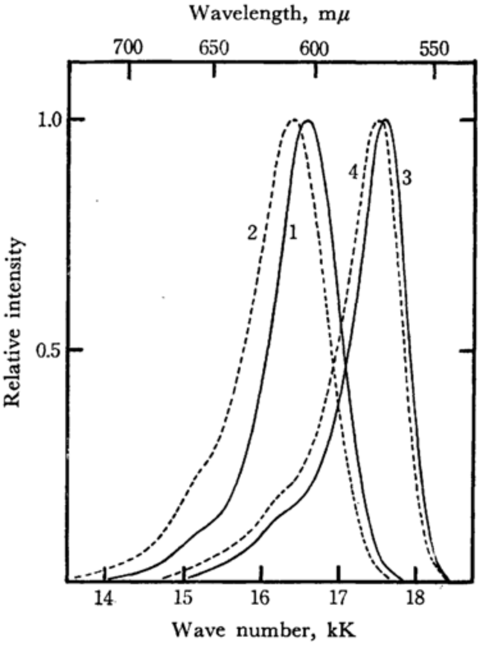

Fig. 5. Phosphorescence spectra of acridine orange and proflavine in glycerine at 77°K.
Excitation wavelength: 470 mμ for proflavine, 500 mμ for acridine orange
Acridine orange: (1) 1.05×10^{-4} M, $\tau=1.80$ sec
(2) 5.94×10^{-3} M, $\tau=1.54$ sec
Proflavine: (3) 5.86×10^{-5} M, $\tau=2.30$ sec
(4) 5.24×10^{-3} M, $\tau=2.10$ sec

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[10] V. Zanker, *Z. Physik. Chem. (Leipzig)*, **200**, 250 (1952).

[11] N. Mataga, This Bulletin, **30**, 375 (1957).

with an increase in the dye concentration. As may be noticed from Fig. 1 for acridine orange, the ESR spectra are presumed to be made up by the superposition of the spectrum due to the triplet state of a monomer and that due to the triplet state of a dimer; the latter, having a D^* value smaller than that of the former, becomes marked with an increase in the dye concentration. In agreement with this argument, in solvents containing water, where acridine dyes form aggregates more easily than in pure organic solvents, this phenomenon is pronounced. A possible explanation for the decrease in the D^* value is the delocalization of two unpaired electrons in a dimer molecule. This theory is, of course, still speculative. If so, however, the resulting increase in the interelectronic separation (r_{12}) may lead to a decrease in the D^* value since:

$D \propto |\langle(r_{12}^2-3z_{12}^2)/r_{12}^5\rangle|$ and $E \propto |(\langle y_{12}^2-x_{12}^2)/r_{12}^5\rangle|$.12)

ESR measurements clarify the nature of the triplet state itself through the magnetic dipolar interaction between unpaired spins, whereas phosphorescence measurements give information regarding the triplet-singlet transition and the energy level of the triplet state. Therefore, phosphorescence measurements have been carried out to establish further details of the triplet state of acridine dyes in connection with their association. As preliminary findings, Fig. 5 shows the phosphorescence spectra of acridine orange and proflavine. In both cases, the maximum of phosphorescence spectra shifts to a longer wavelength with an increase in the dye concentration. The phosphorescence lifetime has been found to be dependent on the dye concentration and on the wavelength (both emission and excitation). Further study is in progress and will be reported elsewhere.

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12) S. A. Boorstein and M. Gouterman, *J. Chem. Phys.*, **39**, 2443 (1963).