Association and Self-quenching of Proflavine in Water

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The association of proflavine (3,6-diaminoacridine) in pH 4 buffer solutions has been investigated by measuring absorption spectra and quantum efficiencies of fluorescence as a function of concentration. The change in the absorption spectrum with concentration was interpreted as due to the formation of a dimer, although there are indications that some higher polymers are formed. The heat of dimerization was calculated from the temperature dependence of the observed extinction coefficient and found to be $\Delta H^\circ = -5{\cdot}5 \pm 0{\cdot}5$ kcal mole^{-1} and the entropy change to be $\Delta S^\circ = -6$ cal mole^{-1} deg. Self-quenching of proflavine was interpreted by a model which assumed both the formation of non-fluorescent dimers in the ground state and the diffusion of excited and ground-state monomers to form an excited dimer incapable of emission (dynamic quenching). The equilibrium constant for dimerization was estimated to be about 500 l. mole^{-1} and the rate constant for pairing about 5×10^{11} l. mole^{-1} sec^{-1}. The heat of dimerization calculated from the temperature coefficient of fluorescence was $\Delta H^\circ = -5{\cdot}5 \pm 1{\cdot}0$ kcal mole^{-1} and is in good agreement with the value obtained from absorption spectrum measurements.

The association of dyestuffs in aqueous and organic solvents has been studied by Rabinowitch and Epstein [1] (thionine and methylene blue in water) and Levshin [2, 3] (several acridine derivatives in organic solvents). Evidence for association (as studied by absorption spectra) and for self-quenching of these dyes appears to begin at about the same concentration and thus led Förster [4] to put into one class (type II) the self-quenching of all dyes in water. Type II self-quenching is assumed to be due entirely to static association (i.e., the formation of non-fluorescent dimers) and Förster cites thionine as a typical case. The self-quenching of many dyestuffs in water cannot be due entirely to static association,[5] since the lifetime of fluorescence usually decreases with increasing concentration. This effect has also been noted by Levshin,[3] especially for proflavine in ethanol. However, such lifetime measurements are sometimes difficult to interpret because of fluorescence reabsorption and re-emission effects; for fluorescein, increasing the concentration actually increases the lifetime.[6] Reabsorption and re-emission of fluorescence can also cause errors in self-quenching measurements unless care be taken to correct for this effect.[7]

The self-quenching of dyes by the Förster mechanism (energy transfer over large distances induced by dipole-dipole coupling) requires considerable overlap between absorption and fluorescence spectra. This mechanism of quenching (type III) should be independent of the viscosity and temperature of the solution.

Collisional quenching has been advanced to explain the self-quenching of aromatic molecules in solution [8] and has been tested [9] by studies on the effect of viscosity of the solvent on the self-quenching constant. Unfortunately, it is not possible to study the effect of viscosity on the self-quenching of dyes by addition of a viscous solvent (e.g., glycerol) since the degree of association and the optical properties are very sensitive to solvent.

Proflavine was chosen for the present study since this dye shows little overlap

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between absorption and fluorescence spectra. This small overlap rules out the possibility of long-distance energy transfer as a mechanism for self-quenching and also eliminates errors due to fluorescence reabsorption followed by re-emission. The optical properties and self-quenching of the free base of proflavine in ethanol and other solvents has been studied by Levshin,[3] who found the very unusual result that the dimer is fluorescent. This paper describes somewhat similar measurements on the proflavine ion in pH 4 water solutions.

EXPERIMENTAL

Proflavine (3,6-diaminoacridine hemisulphate hemihydrate from the Aldrich Chemical Co.) was purified by cooling the saturated solution in water to 0°, filtering the crystals, washing with ice water followed by ether and drying in a vacuum oven for 4 days. Analysis of the crystals suggested the formula $(C_{13}H_{12}N_3)_2SO_4 \cdot 3H_2O$ (found: C = 55 %, H = 5·3 %, S = 5·7 %; calc.: C = 54·7 %, H = 5·26 %, S = 5·61 %). The free base was also prepared from the purified acid salt, recrystallized from a water+methanol mixture (70/30), and dried in a vacuum oven at 70°.

The buffer solutions used were 0·05 M acetate+acetic acid from pH 4 to 5, 0·05 M acetate+HCl from pH 1 to 5, and 0·01 M potassium hydrogen phthalate, pH 4. Most work was done in the acetate buffers in which solutions as strong as $3{\cdot}5 \times 10^{-2}$ M could be prepared. Solutions more concentrated than 4×10^{-4} M could not be prepared in the phthalate buffer. Adsorption of proflavine on glass surfaces was found to occur at all concentrations. Care was therefore taken, between measurements, to wash the cuvettes and other glassware with dilute nitric acid followed by distilled water. Errors due to adsorption of the dye on the pipettes were less than 4 % as indicated by the agreement between extinction coefficients of solutions made by weighing the components and by dilution with pipettes from more concentrated solutions. All solutions were made up in low actinic glassware, stored in dark bottles and used within 24 h. The fluorescence intensities and extinction coefficients were independent of time over a 12-h period and the solutions were therefore considered to have attained equilibrium. Despite every precaution, measurements in the region 10^{-5}-10^{-6} M were not reproducible to better than about ± 5 %.

Absorption spectra were measured with a Cary model 14 spectrophotometer using the thermostatically controlled cuvette holder. Possible errors in the extinction coefficient due to fluorescence of the sample were shown to be negligible. Fluorescence spectra were measured with a modified Aminco-Keirs spectrofluorimeter [10] in which the solutions could be excited and observed from the same side of a rectangular quartz cuvette. The exciting wavelength used was 445 mμ. Fluorescence quenching measurements were made by observing the total fluorescence with a Dumont 6291 photomultiplier. The solutions were illuminated at 45° and observed perpendicularly to the cuvette face. Corrections for the incomplete absorption of the exciting light (in this case the Hg 436 mμ line, isolated with a second-order interference filter and a Corning no. 3391 glass filter) were made as described previously.[7] A Corning no. 3387 filter was used to eliminate any stray light reaching the photomultiplier. Air was not removed from the solutions since dissolved oxygen was found not to measurably affect the fluorescence intensity.

RESULTS

The absorption spectrum of proflavine was measured over a wide concentration range in pH 4 acetate buffer and in water. Deviations from Beer's law appeared above 5×10^{-5} M and the 444 mμ peak shifted to shorter wavelengths (fig. 1 and 2). Below 5×10^{-5} M the molar extinction coefficient was $4{\cdot}1 \times 10^4$ in both acetate and phthalate buffers. This value is considerably higher than that given by Craig and Short [11] ($\varepsilon \approx 2{\cdot}8 \times 10^4$, concentration not stated) and Millich and Oster [12] ($\varepsilon = 3{\cdot}34 \times 10^4$ at $\leq 6 \times 10^{-5}$ M). Both the acid salt and the purified base dissolved in pH 4 acetate gave the same extinction coefficient. The change in the extinction

coefficient with concentration seems to be consistent with the formation of a dimer at all concentrations up to about 10^{-2} M.

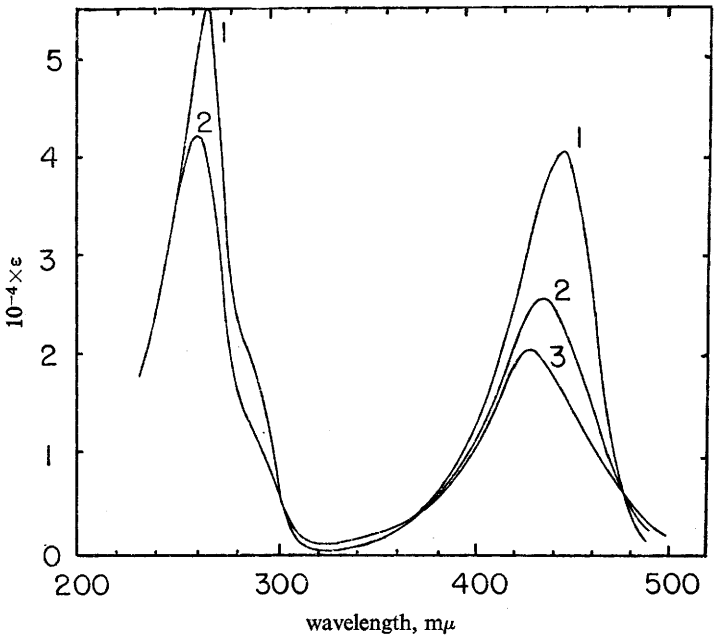

FIG. 1.—Absorption spectrum of proflavine in pH 4 acetate buffer at 25°; 1 = $3{\cdot}5\times10^{-6}$ M, 2 = $3{\cdot}5\times10^{-3}$ M, 3 = $3{\cdot}5\times10^{-2}$ M.

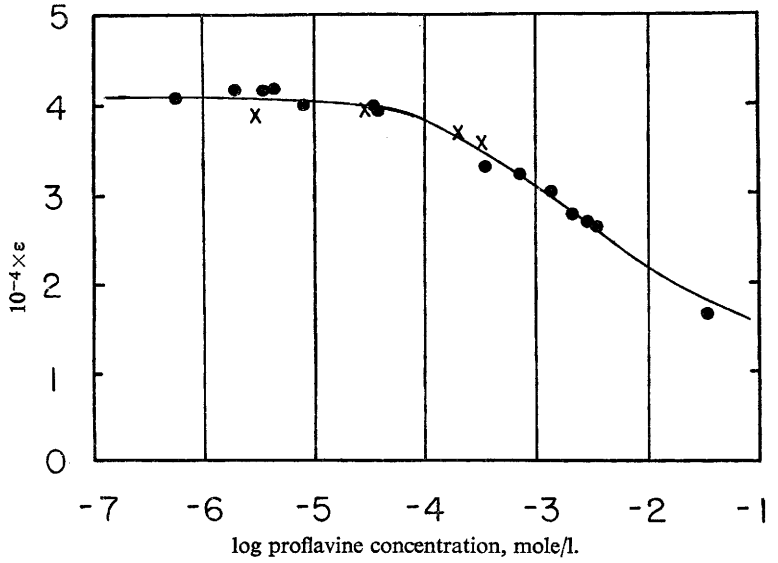

FIG. 2.—Molar extinction coefficient of proflavine as a function of concentration (25°); × = water. ● = pH 4 acetate buffer.

Thus, if c_m = monomer concentration, c_d = dimer concentration, $K = c_d/(c_m)^2$ = equilibrium constant for dimer formation. Then $\varepsilon = \alpha(\varepsilon_m - \varepsilon_d/2) + \varepsilon_d/2$, where

$\alpha = c_m/C = [(1+8CK)^{\frac{1}{2}}-1]/4CK$, C = total dye concentration, ε = total extinction coefficient at λ, ε_m = monomer extinction coefficient at λ, ε_d = dimer extinction coefficient at λ.

The experimental points on fig. 2 are well fitted by $K = 500$ l. mole^{-1} and ε_d ($\lambda = 445$ mμ) $= 3\times10^4$, although there is some latitude in the choice of these parameters. The absorption spectrum of the dimer (fig. 3) was obtained from eqn. (1) using $K = 500$ l. mole^{-1} and computing ε_d at each wavelength. Two

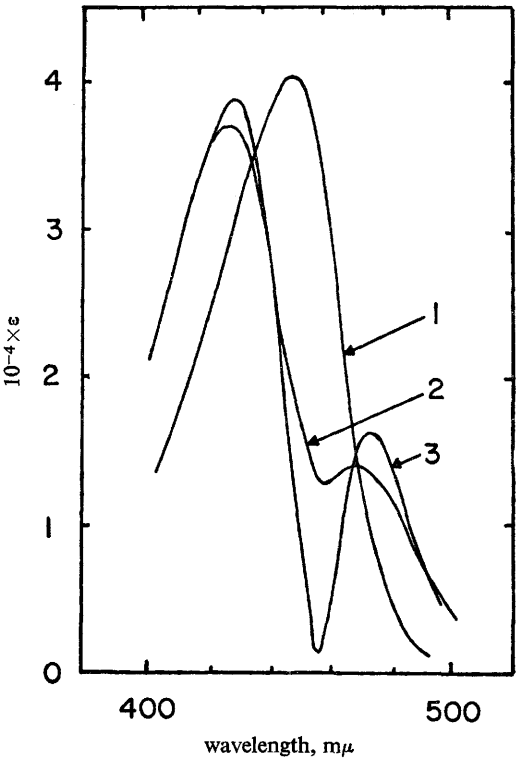

FIG. 3.—Absorption spectra of the monomer and dimer in pH 4 buffer (25°); 1 = monomer. 2 = $3{\cdot}5\times10^{-2}$ M (dimer), 3 = $3{\cdot}5\times10^{-3}$ M (dimer).

bands are obtained, one at shorter and the other at longer wavelengths than the monomer bands which is in agreement with the ideas of Förster.[13] At higher concentrations the dimer spectrum changes shape, possibly as the result of the formation of higher polymers.

The absorption spectrum of concentrated solutions of proflavine showed increased absorption with rising temperatures. Since dilute solutions did not show this effect, the change was considered to be due to the formation of monomers from dimers. The heat $\Delta H°$ of formation and the entropy $\Delta S°$ of dimerization may be calculated from the change in optical density with temperature as follows.

Since temperature changes are small, the integrated form of the van't Hoff equation may be used to determine $\Delta H°$:

$$\ln\left(\frac{K(T)}{K(298)}\right) = -\left(\frac{\Delta H°}{R}\right)\left(\frac{1}{T}-\frac{1}{298}\right),$$

where K is the equilibrium constant of dimerization. Since the dye concentration C is independent of temperature:

$$\frac{K(T)}{K(298)} = \frac{1-\alpha(T)}{\alpha(T)^2} \cdot \frac{\alpha(298)^2}{1-\alpha(298)},$$

where

$$\alpha(T) = c_m(T)/C.$$

Thus

$$\ln\left(\frac{[1-\alpha(T)]}{\alpha(T)^2}\right) = \text{constant} - \left(\frac{\Delta H^\circ}{R}\right)\left(\frac{1}{T} - \frac{1}{298}\right). \quad (2)$$

The optical density (OD) of the solution may be obtained from eqn. (1):

$$\text{OD} = \varepsilon_m c_m + (\varepsilon_d/2)(C - c_m)$$

for a 1-cm cuvette. Thus

$$\text{OD}(T) - \text{OD}(298) = [c_m(T) - c_m(298)](\varepsilon_m - \varepsilon_d/2).$$

Therefore

$$\alpha(T) = \left[\frac{\text{OD}(T) - \text{OD}(298)}{C(\varepsilon_m - \varepsilon_d/2)}\right] - \alpha(298).$$

$\alpha(T)$ is then substituted into eqn. (2) to obtain ΔH°. The value of ΔH° at concentrations $3{\cdot}5\times10^{-2}$, $3{\cdot}5\times10^{-3}$ and $3{\cdot}5\times10^{-4}$ M over a temperature range of 273° to 323°K was found to be $-5{\cdot}5\pm0{\cdot}5$ kcal mole^{-1}. With $K = 500$ l. mole^{-1}, ΔS° was calculated to be approximately -6 cal mole^{-1} deg. The entropy change is larger than can be accounted for by simple pairing in solution and this fact, together with the usual concentration dependence of the dimer spectrum, suggests that higher polymers might be formed.

Fluorescence spectra (fig. 4) and quantum efficiencies of fluorescence of $3{\cdot}5\times10^{-4}$ M solutions of proflavine were measured from pH 1 to 5. These spectra have been corrected for instrumental effects and $Q(\mu^{-1})$ is the relative number of quanta sec^{-1} steradian^{-1} $(\mu^{-1})^{-1}$ at the wavenumber μ^{-1}. From the marked decrease in fluorescence intensity at pH 1·5, it was concluded that the addition of a second proton was occurring with a $K_a \approx 10^{-1{\cdot}5}$ mole l.$^{-1}$ (298°K). The equilibrium constant for the addition of a proton to the ring nitrogen [14] is $K_a' = 10^{-9{\cdot}6}$ mole l.$^{-1}$. Thus the percentages of the various species present in a pH 4 buffer solution may be calculated: single ionized = 99·7 %, doubly ionized = 0·3 % and non-ionized = $3{\cdot}3\times10^{-4}$ %.

The self-quenching of the singly-charged ion of proflavine at 0, 25 and 50° was investigated over the concentration range $3{\cdot}5\times10^{-3}$ to $1{\cdot}4\times10^{-6}$ M in 0·05 M acetate buffer (pH 4) and from $3{\cdot}5\times10^{-4}$ to $3{\cdot}5\times10^{-6}$ M in 0·1 M phthalate buffer (pH 4). The results plotted on fig. 5 have been corrected for incomplete absorption of the exciting light at low ($<8\times10^{-5}$ M) concentrations. Fluorescence re-absorption—re-emission corrections have not been made since the overlap between absorption and fluorescence spectra is small (see fig. 4). That re-absorption was not important was supported by the fact that the shape of the fluorescence spectrum did not change detectably when the concentration was increased from $3{\cdot}5\times10^{-6}$ to $3{\cdot}5\times10^{-3}$ M.

The absolute quantum efficiency of fluorescence of proflavine at low concentrations in the pH 4 acetate buffer was measured with the rhodamine B quantum counter [7] and found to be $0{\cdot}34 \pm 0{\cdot}02$. The quantum efficiencies Q on fig. 5 are based on this figure.

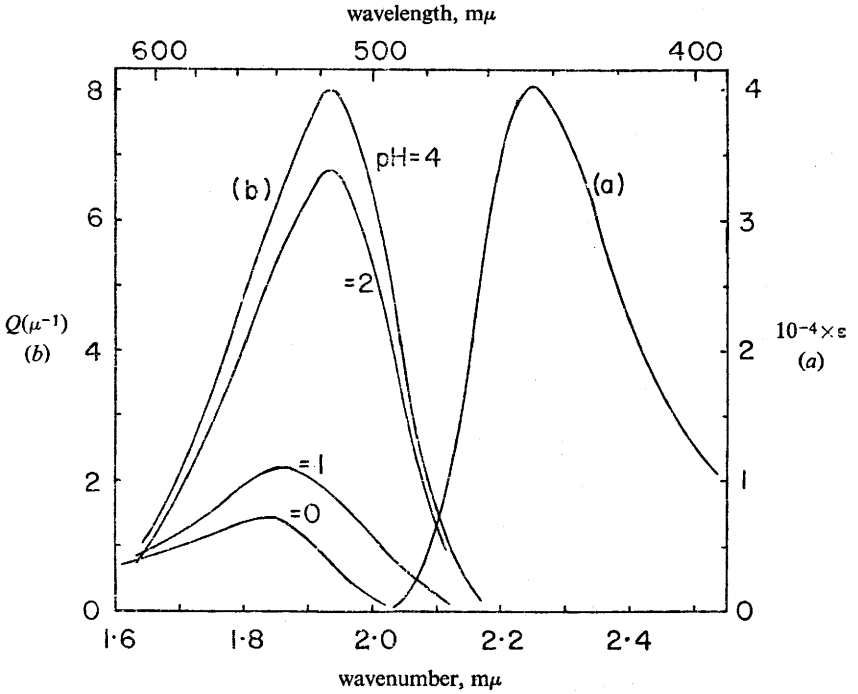

FIG. 4.—Absorption spectrum (*a*), and fluorescence spectra (*b*), of proflavine at 25°.

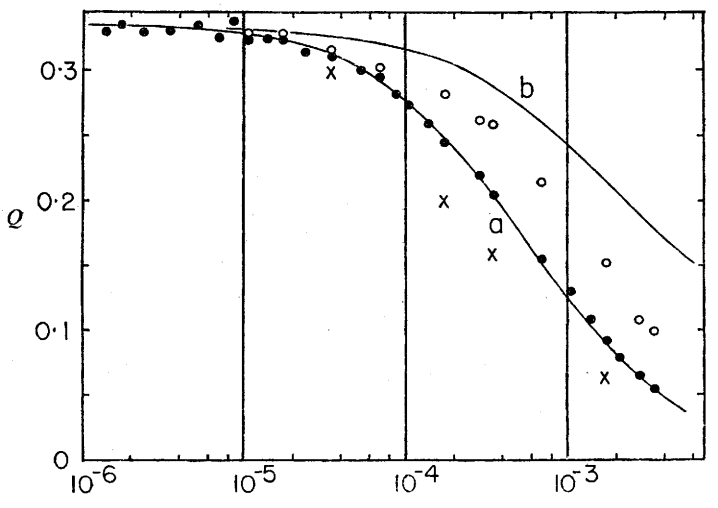

FIG. 5.—Self-quenching of proflavine in pH 4 buffer: × = 0°, ● = 25°, ○ = 50° *a*, calc. using $K = 500$ and $k_4' = 10^3$; *b*, calc. curve $K = 500$.

DISCUSSION

The concentration and the temperature dependence of the fluorescence efficiency of proflavine are similar to those found for many other dyestuffs in water.* Of the possible quenching mechanisms proposed by Förster, type III (energy transfer) is inadmissible because overlap between absorption and fluorescence spectra is small. Thus, using Förster's equation [15] for energy transfer, it was found that the critical concentration for proflavine (where the fluorescence has dropped to one quarter of its value in dilute solution) was approximately $1{\cdot}5 \times 10^{-2}$ M. This is 10 times larger than is observed. Nor is type II quenching capable of explaining the observations, as curve *b* of fig. 5 shows. Curve *b* has been calculated assuming the formation of non-fluorescent dimers from monomers using $K = 500$ l. mole^{-1} (as measured by absorption spectroscopy). It therefore seems necessary to include diffusional quenching as the mechanism for self-quenching, and table 1 lists the possible rate processes. The proflavine ion, like its parent, the acridinium ion,[16]

TABLE 1.—DIMER MODEL FOR THE SELF-QUENCHING OF PROFLAVINE

	rate
absorption:	
$P + h\nu \rightarrow P^*$	$I(\varepsilon_m/\varepsilon)\alpha$
$P_2 + h\nu \rightarrow P_2^*$	$I(\varepsilon_d/\varepsilon)\frac{1}{2}(1-\alpha)$
fluorescence:	
$P^* \rightarrow P + h\nu_F$	$k_1 P^*$
radiationless transitions:	
$P^* \rightarrow P$ + heat, etc.	$k_2 P^*$
$P_2^* \rightarrow P_2$ + heat, etc.	$k_3 P_2^*$
diffusion processes:	
$P + P^* \rightarrow P_2^*$	$k_4 P P^*$
$P_2 + P^* \rightarrow P_2^* + P$	$k_6 P_2 P^*$
$P_2^* \rightarrow P^* + P$	$k_5 P_2^*$
$P_2^* + P \rightarrow P_2 + P^*$	$k_7 P P_2^*$

Definitions of symbols:

P and P_2 are the concentrations of monomer and dimer (mole/l.);
I = intensity of the exciting light.

probably remains protonated during the lifetime of its excited state and thus it is not necessary to consider the excited free base in the reaction scheme. Note that association in the ground state has been allowed for in the first two equations by writing α = the fraction of the dye as monomers and $(1-\alpha)$ = the fraction of the dye as dimers. Using the steady-state approximation, then

$$I[(\varepsilon_m/\varepsilon)\alpha + \theta(\varepsilon_d/\varepsilon)\tfrac{1}{2}(1-\alpha)] = P^*[(k_1+k_2+k_4P+k_6P_2) - \theta(k_4+k_6P_2)], \qquad (3)$$

where P = monomer concentration (mole/l.), P_2 = dimer concentration (mole/l.), $\theta = (k_5+k_7P)/(k_3+k_5+k_7P)$. By extrapolation to zero concentration ($\alpha = 1$ and $P = P_2 = 0$), eqn. (3) becomes

$$I = P_0^*(k_1+k_2). \qquad (4)$$

* Strong self-quenching of proflavine (in pH 4 buffer) has been reported [12] at concentrations where we found weak self-quenching.

Substitution of eqn. (4) into (3), writing $P_0^*/P^* = Q_0/Q = 0{\cdot}34/Q$, and noting that $1-(\varepsilon_d/\varepsilon)\frac{1}{2}(1-\alpha) = (\varepsilon_m/\varepsilon)\alpha$ gives

$$(0{\cdot}34/Q)[(\varepsilon_m/\varepsilon)\alpha(1-\theta)+\theta] = 1+(1-\theta)(k_4'P+k_6'P_2), \quad (5)$$

where the prime denotes division by (k_1+k_2). The lifetime of fluorescence τ is given by $1/(k_1+k_2)$ at low concentrations.

It is assumed that radiationless transitions in the excited dimer are fast compared with the rate of break-up of the dimers (i.e., $k_3 \gg k_5+k_7P$, whence $\theta = 0$) and thus

$$(0{\cdot}34/Q)(\varepsilon_m/\varepsilon)\alpha = 1+k_4'P+k_6'P_2, \quad (6)$$

where Q = quantum efficiency of fluorescence. Eqn (6) may be rewritten as

$$(0{\cdot}34/Q)(\varepsilon_m/\varepsilon)\alpha = 1+[k_4'+(k_6'/2)(1-\alpha)]C. \quad (6')$$

Now it was found that the l.h.s. of eqn. (6′) (see table 2) was proportional to C (the total dye concentration) which implies that $k_6(1-\alpha)/2$ is small compared with k_4' and may be neglected. Thus

$$(0{\cdot}34/Q)(\varepsilon_m/\varepsilon)\alpha = 1+k_4'C. \quad (7)$$

TABLE 2.—TEST OF EQN. (7) (AT 25°C)

conc. of dye (C) moles l.$^{-1}$	$\frac{0{\cdot}34}{Q}\left(\frac{\varepsilon_m}{\varepsilon}\right)\alpha/(1+k'C)$		
	$k_4' = 1000$	$k_4' = 900$	$k_4' = 800$
0	1	1	1
$0{\cdot}5\times10^{-3}$	1	1	1
$1{\cdot}0\times10^{-3}$	·9	1	1
$1{\cdot}5\times10^{-3}$	·9	1	1
$2{\cdot}0\times10^{-3}$	·9	·9	1
$2{\cdot}5\times10^{-3}$	·8	·9	·9
$3{\cdot}0\times10^{-3}$	·8	·9	1
$3{\cdot}5\times10^{-3}$	·8	·8	·9

Curve *a* of fig. 5 has been calculated from eqn. (7) using $K = 500$ l. mole^{-1} and $k_4' = 10^3$, although a fairly wide range of values can be found which give an equally good fit. The value $K = 500$ l. mole^{-1} has been chosen here since this was the estimated figure obtained from absorption spectra measurements. The lifetime of proflavine calculated from Förster's lifetime equation [17] is $\tau = 2\times10^{-9}$ sec ($\tau = \tau_e Q_0$ where $\tau_e = 5{\cdot}8\times10^{-9}$ sec and $Q_0 = 0{\cdot}34$). Thus the diffusion rate constant k_4 is approximately 5×10^{11} l. mole^{-1} sec^{-1}, a figure which is larger than the accepted rate of encounter formation in liquids by diffusion and may indicate that self-quenching can occur over distances greater than the encounter distance.

The temperature dependence of the fluorescence intensity becomes strongly positive above about 10^{-4} M and indicates that dimers in the ground state are broken up, as the temperature is increased, into monomers capable of fluorescing when excited. It is possible to obtain α as a function of temperature from eqn. (7), thus,

$$\alpha(T) = (1+k_4'C)(\varepsilon/\varepsilon_m)_T(Q(T)/0{\cdot}34),$$

where $Q(T)$ = fluorescence efficiency at the temperature T and k_4' is assumed independent of temperature.

These values of $\alpha(T)$ were then substituted into eqn. (2) and ΔH° was calculated to be $-5{\cdot}5\pm1{\cdot}0$ kcal mole^{-1} at concentrations between 10^{-4} and $3{\cdot}5\times10^{-3}$ M, in good agreement with the value obtained from optical density measurements.

The work of Levshin [3] on the free base of proflavine in ethanol and other organic solvents is strikingly different from our results on the singly-charged ion in water. They found the neutral dimer is capable of fluorescing, whereas our charged dimer is not. Furthermore, Levshin found increasing quenching with increasing temperature, while the reverse was found here. There was little evidence for the polymerization of proflavine to micelles as was found for thionine; [18] however, it is possible that small groups of molecules are formed, each cluster containing perhaps 3, 4, or 5 molecules. Calculations assuming the formation of trimers were attempted and appeared to fit the experimental data quite well provided reasonable estimates were made for the additional rate constants introduced.

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