uents, acetate and benzoate (Table I, 4–8).

This investigation is being extended to include the salts of iodine(I) coördinated with the picolines, quinoline and other amines, as well as analogous compounds of bromine(I).

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY POLYTECHNIC INSTITUTE OF BROOKLYN]

Photoreduction of Acridine Dyes[1,2]

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RECEIVED SEPTEMBER 5, 1958

A number of acridine dyes in the presence of allylthiourea are reduced to their leuco forms on irradiation with blue light. Those acridines which have amino substituents in both the 3- and 6-position undergo photoreduction rapidly. Another group of acridines undergoes photoreduction at one tenth the rate of the first and a third group exhibits no reactivity. A correlation exists between phosphorescence of the dyes and their ability to undergo photoreduction. The detailed kinetics of photoreduction of proflavin at its *p*H for maximum rate, namely *p*H 4, coupled with flourescence studies showed that: (a) the reduction proceeds through a long-lived excited state, (b) the transition from the first singlet excited state to the long-lived state is induced by dye molecules in the ground state, (c) the inductive forces of interaction act over distances of 500 Å.

Introduction

An examination of a wide variety of water-soluble dyes shows that dyes of only a few families are susceptible to photoreduction. Kinetic studies of photoreduction of the fluoresceins,[3,4] of the thiazines[5] and of the basic triphenylmethanes in the bound state[6] have shown that the reaction proceeds *via* a long-lived excited state. The acridine dyes are noted for their resistance to reduction in the dark.[7,8] Acriflavin, however, under certain conditions, does undergo photoreduction.[9]

The present paper is concerned with the photoreductive properties of a large number of acridine dyes in order to investigate the generality of the role played by the long lived state and to correlate structure, luminescence and photoreducibility. As will be shown, the kinetics of photoreduction of proflavin reveals a number of unusual features, in particular, energy transfer processes involving action over extremely long distances.

Experimental

A. Materials.—The practical grade of 3,6-diaminoacridine, obtained as the sulfuric acid salt (mol. wt. 307, Eastman Organic Chemicals), was purified by treating an aqueous solution with activated charcoal. The solution was then concentrated, chilled overnight, filtered and rinsed with a little ethyl ether. The precipitate was air-dried and then dried overnight in a vacuum oven at 70°.

The following persons and institutions graciously donated purified samples of acridine dyes, listed in Table I: Dr. Adrien Albert, The Australian National University, Canberra, A. C. T. (compounds 1b, 1d, 1e, 2b, 2c, 3a, 3d, 3f and 3g); Dr. Peter P. H. De Bruyn, the University of Chicago, Illinois (compounds 2a, 2d, 3b and 3e); General Aniline and Film Corp. (compound 1g); Abbott Labs. (compound 2e); and Sterling-Winthrop Research Institute (compound 3c). Compounds 1c and 1f are commercially available (National Aniline) and were used as received.

Allylthiourea (Eastman) was decolorized with charcoal and recrystallized from acetone. Ascorbic acid (Hoffmann-LaRoche Inc.) was U. S. P. grade. All other reagents were C.P. grade. Helium (Airco) was used to flush oxygen from reactant solutions.

B. Procedures.—A representative photoreductive solution consisted of proflavin (10^{-6} to 10^{-4} mole/liter), allylthiourea (10^{-2} mole/liter), in water, buffered to *p*H 4.0 (10^{-2} mole/liter sodium dihydrogen phosphate or potassium hydrogen phthalate, brought to the desired *p*H with sodium hydroxide or hydrochloric acid). The solutions were deaerated with helium 15 minutes prior to and then during illumination, in cells of 5 × 5 cm. cross-section and 1 cm. thickness. The period of deaeration proved to be adequate as judged by the fact that the initial rates of photoreduction were unaffected by longer periods.

TABLE I

THE ACRIDINE DYES AND THEIR SPECTRA ABSORPTION MAXIMA[a]

Class I. 1a. 3,6-Diaminoacridine, proflavin[b] (444); 1b. 3,6-diamino-10-methylacridinium chloride, euflavin[b] (452); 1c. 2,7-dimethyl-3,6-diaminoacridine, acridine yellow[b] (442); 1d. 2,7-dimethoxy-3-6-diaminoacridine (445); 1e. 3,6-diamino-4,5-dimethylacridine (452); 1f. 3,6-bis-(dimethylamino)-acridine, acridine orange[b] (494); 1g. 2,7,9-trimethyl-3,6-diaminoacridine (437).

Class II. 2a. Atebrin[b] (445, 425, 340); 2b. 9-(β-hydroxyethylamino)-acridine (433, 410, 390;) 2c. N,N′-bis-(9-acridyl)-ethylenediamine (440,[c] 410); 2d. 10-methylacridinium chloride (410, 358, 340); 2e. 9-(*p*-cyclohexyloxyphenyl)-10-methylacridinium chloride, acrizan R chloride[b] (430, 360)

Class III. 3a. 2-Aminoacridine (460, 370); 3b. 3-aminoacridine (460, 370); 3c. 9-aminoacridine (425, 400, 380); 3d. 2,6-diaminoacridine (490, 368); 3e. 2,7-diaminoacridine (490, 368); 3f. 3,7,9-triaminoacridine (445); 3g. 9,9′-bis-(36,-diamino-10-methylacridinium)-dinitrate, bis-trypaflavin[b] (475).

[a] All spectral maxima (in mμ) and the photoreductive property classification refer to solutions buffered in the range of *p*H 2.5–4. [b] The trivial name of the dye is given. [c] A shoulder and not a peak is present.

For relative rate measurements the sample cell was illuminated with white light from a 500-watt tungsten lamp TDC slide projector (stabilized with a Sola constant-voltage transformer) at a distance of 15 cm., through a Corning No. 3389 near ultraviolet cut-off filter. Interference filters of 100 Å. band width (Photovolt Corp., N. Y. C.) were employed directly in front of an RCA No. 931-A photomultiplier tube. With each dye an appropriate interference filter was chosen which had its maximum transmission at the maximum of the absorption band of the dye. The relative transmittances of the solutions were measured

(1) This paper represents a part of the dissertation submitted by Frank Millich to the faculty of the Graduate School of the Polytechnic Institute of Brooklyn in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(2) This was supported by the United States Air Force through the Air Force Office of Scientific Research of the Air Research and Development Command under Contract No. AF 18(600)-1182 and under Contract No. AF 19(604)-3065.

(3) G. Oster and A. H. Adelman, THIS JOURNAL, **78**, 913 (1956).

(4) A. H. Adelman and G. Oster, *ibid.*, **78**, 3977 (1956).

(5) G. Oster and N. Wotherspoon, *ibid.*, **79**, 4836 (1957).

(6) G. Oster and J. S. Bellin, *ibid.*, **79**, 294 (1957).

(7) A. Albert, "The Acridines," Edward Arnold and Co., London, 1951.

(8) R. M. Acheson and L. E. Orgel, "Acridines," Interscience Publishers, Inc., New York, N. Y., 1956.

(9) G. Oster, *Trans. Faraday Soc.*, **47**, 660 (1951).

with an Aminco photometer unit and recorded on a Leeds and Northrup Speedomax Type G recorder.

Absolute values of the quantum yield of photoreduction of proflavin were obtained from initial slopes of absorbance *versus* time curves and a determination of the quantum output of the light source. Absolute quantum yield was evaluated, as previously described[3] with a calibrated thermopile (Eppley), and with monochromatic illumination by using an interference filter with maximum transmission at 440 ± 5 mμ positioned between the lamp and the sample cell. White light was employed for most of the rate studies for convenience since the rates were of an order of magnitude higher than those with monochromatic light. It was assumed that the quantum yields are independent of the wave length of incident light.

The procedure for determination of fluorescence intensity by frontal observation has been previously described.[10] The measurements were made in an Aminco light scattering apparatus using a cylindrical sample cell placed at the center of a rotating phototube mount and the cell was wrapped with heating tape.

Absorption spectra were determined in a Beckman DU spectrophotometer.

Ubbelohde viscometers were used for the determination of viscosities of the aqueous glycerol solutions.

The phosphorescence survey was carried out visually in a dark room using ultraviolet light (365 mμ) and glycerol solutions at the temperature of acetone–Dry Ice (−90°).

Results

Not all acridines undergo photoreduction under the conditions which we employed. The nineteen acridines shown in Table I can be grouped into three classes according to their relative rates of photoreduction in the *p*H range of 1 to 6. The members of class I, which are characterized by having amino substituents in both the 3- and 6-position, photoreduce rapidly. The members of class II photoreduce at best at $^1/_{10}$th the rate of those of the first class. This second class is constituted by two structural types: the members either have 9-alkylamino substituents, or they are quaternized at the 10-position. One of the latter, compound 2e, bleaches slowly in the dark in the presence of allylthiourea after oxygen is removed, although light accelerates the bleaching. Class III groups those acridines which do not undergo photoreduction.

Among reagents which act as hydrogen donors for the photo-excited acridinium dye, allylthiourea and ascorbic acid seem to be the best. These two compounds are equivalent as regards the general shapes of the kinetic curves of fading, the initial rates and the *p*H dependence. Ascorbic acid differs from allylthiourea, however, in that the reaction is slowly reversed in the dark without the admittance of oxygen. In the presence of allylthiourea, the leuco-product of the photoreduction of proflavin can be reoxidized by the admittance of air at an early stage- but, under prolonged illumination it suffers an irreversible change such that it is then no longer reoxidizable by air.

The kinetics of photoreduction show a strong dependence upon the concentration of hydrogen ion. At *p*H 4 the absorbance *versus* time curve for proflavin in the presence of allylthiourea shows a maximum rate and is second order with respect to the concentration of dye. At *p*H values greater than 4 the reaction is less proficient, both with regard to the initial slope and with regard to the ultimate level to which the reaction advances. At *p*H values less than 4 the curves show progressively greater development of an "S" shape. Euflavin and acridine orange also give qualitatively similar results in that there are *p*H maximum regions. In general, for the remaining dyes of class I and class II the photoreductive rates increase with lowering *p*H values, but the occurrence of maximum was not investigated.

Because of the complexity of the kinetics of photoreduction, the detailed study was restricted to just one member dye of class I, namely, proflavin. Solutions containing allylthiourea and buffered to *p*H 4 were used exclusively since maximum rates and linear initial slopes are obtained. The rate of photoreduction is linearly dependent upon the intensity of monochromatic illumination of frequency corresponding to the frequency of maximum absorption of the dye. The quantum yield for a solution of 2×10^{-5} *M* proflavin and 1×10^{-2} *M* allylthiourea concentration is 1.0×10^{-2} mole/einstein.

The dependence of quantum yield upon proflavin concentration, depicted in Fig. 1 for several different concentrations of reducing agent, follows the unusual relation of increasing with increasing concentration of the dye. This dependence fits an equation of the form: rate $= a(\mathrm{D})K/[b + a(\mathrm{D})]$, where (D) is the molar concentration of proflavin, and a, b and K are constants. When 1/rate is plotted *versus* 1/(D) a series of straight lines is obtained with an intercept equal to $1/K$, and a slope equal to b/aK. The ratio of constants b/a is obtained, therefore, by dividing the value of the slope by that of the intercept. This ratio is dependent upon the concentration of allylthiourea, and the limiting value of a/b at zero concentration of reducing agent is 4.0×10^4 l./mole.

In Fig. 2 is shown, in a reciprocal plot, the dependence of rate of photoreduction upon reducing agent concentration, (A). For relatively low values of (A) the rate is given by rate $= c(\mathrm{A})K/[d + c(\mathrm{A})]$, where c, d and K are constants. Using the slope and extrapolated intercept of the straight line region, the ratio d/c is evaluated and is found to equal 2.5×10^{-4} mole/liter. At high concentrations of allylthiourea a deviation occurs in such direction as to indicate an enhanced photoreductive process.

The rate of photoreduction is retarded by aromatic compounds, such as quinone, hydroquinone and aniline. Figure 3 shows that a concentration of about 10^{-5} mole/liter of *m*-phenylenediamine diminishes the quantum yield of photoreduction to one half of its original value.

B. Absorption Spectra.—Proflavin in aqueous solution at *p*H 4 shows no deviation from Beer's law with increasing concentration as regards wave length distribution or the molar absorbance index, over a range of concentrations from 2×10^{-6} to 6×10^{-5} *M*. The value of the index at *p*H 4 is 33,400, based on a molecular weight of 307. It is reported that proflavin does not show 10% deviation from Beer's law at *p*H 4 up to a concentration range of 4.5×10^{-4} *M* (ref. 7, p. 111). Listed in Table I are the wave lengths of maximum absorption between 3000 and 6000 Å. for the mono-protonated species of the acridine dyes. The spectra were ob-

(10) G. Oster and Y. Nishijima, THIS JOURNAL, **78**, 913 (1956).

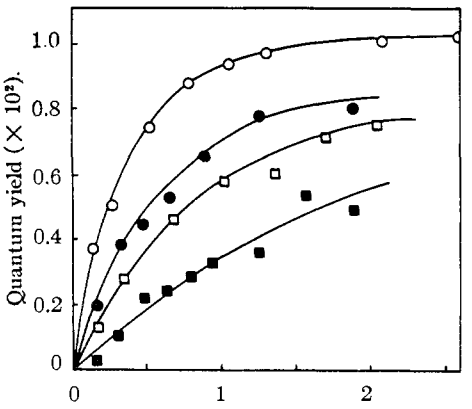

Fig. 1.—Dependence of rate of photoreduction of proflavin on dye concentration. Allylthiourea concentration: 10, 7, 4 and 0.4 millimolar for open circles, closed circles, open squares and closed squares, respectively.

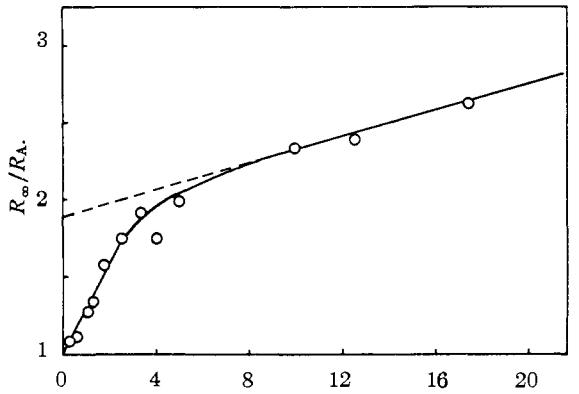

Fig. 2.—Reciprocal of rates, relative to that at infinite reducing agent concentration, as a function of reciprocal allylthiourea concentration.

served in aqueous solution buffered between *p*H's of 2.5 and 4.0. In the ultraviolet region proflavin absorbs at 260 mμ. Although all of the acridines have a strong absorption band in the range of 260 to 290 mμ, these bands are not reported.

C. Fluorescence and Fluorescence Quenching.—A qualitative survey of the acridine dyes shows that the mono-protonated form at *p*H 4 fluoresces well at room temperature in all cases except compounds 2e and 3g. The fluorescence of these latter two dyes is only slightly improved in glycerol solution; however, both dyes fluoresce well in glycerol solution as the temperature is reduced. In general, there is a change of fluorescence spectrum only at values of *p*H in the neighborhood of the pK_a's of each dye, the mono-protonated form usually fluorescing the strongest.

Proflavin, at *p*H 4, has a rather wide fluorescence spectrum with a maximum at about 506 mμ. This band does overlap the absorption band which has its maximum at 444 mμ, but only to an ordinary degree. Proflavin fluorescence, which is green in the *p*H range from 2 to 9, changes to weak yellow at *p*H greater than ten and changes to yellow and gold at *p*H less than one.

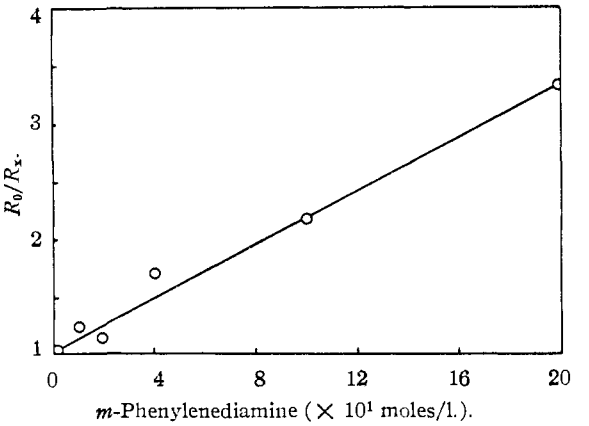

Fig. 3.—Reciprocal of rates, relative to that in absence of retarder, as a function of *m*-phenylenediamine concentration.

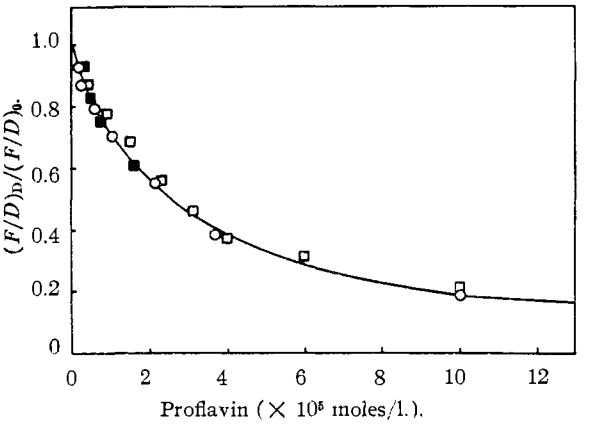

Fig. 4.—Self-quenching of fluorescence: open circles, in water (25, 45 and 65°, superposed); open squares, in glycerol–water (viscosity 6.45 centipoise); closed squares, in glycerol–water (viscosity 111 centipoise).

Three different studies of the quenching of fluorescence of proflavin at *p*H 4 were made: (1) self-quenching, (2) quenching by potassium iodide and (3) quenching by allylthiourea. In addition, since *m*-phenylenediamine was shown to be a very efficient retarder of the rate of photoreduction, its effect upon the fluorescent species was investigated. At six different concentrations up to 4×10^{-4} *M*, *m*-phenylenediamine proved to have no effect upon the fluorescence efficiency of proflavin at 2×10^{-6} *M*. concentration.

1. Self-quenching.—The specific fluorescence of proflavin at *p*H 4 is very strong in dilute solution (10^{-6} *M*.). However, the fluorescence is quenched by increasing concentration of dye at remarkably low concentration from which an average distance of separation of dye molecules of 500 Å. can be calculated. Figure 4 shows the dependence of specific fluorescence, $F/(D)$, relative to that at infinite dilution, $(F/(D))_0$, on concentration of dye (D). This behavior contrasts sharply with that of most other dyes. Fluorescein, for example, at *p*H 7, shows little diminution of specific fluorescence until concentrations of 10^{-4} *M* are exceeded. The self-quenching of proflavin fluorescence was studied in aqueous glycerol solutions of viscosity of 6.46 and 111 centipoise, both at *p*H 4, and proved to be in-

dependent of viscosity. Aqueous solutions were investigated at 25, 45 and 65° and the fluorescence quenching was found to be independent of temperature. One obtains a straight-line relationship by plotting the reciprocal relative specific fluorescence, $(F/D)_0/(F/D)_D$ *versus* the molar concentration of proflavin. The value of the slope of such a plot is 3.6×10^4 liters/mole.

2. Fluorescence Quenching by Potassium Iodide.—The quenching of fluorescence of proflavin by potassium iodide was performed at two different concentrations of the dye since it was reasoned that as the population of the fluorescent species is diminished by self-quenching at higher dye concentration the subsidiary quenching agent would become apparently less effective. An analogy to this situation is that of the quenching of mercury resonance radiation by a mixture of two gaseous quenchers, hydrogen and nitrogen.[11] This effect is indeed present as shown in Fig. 5. The limiting value of

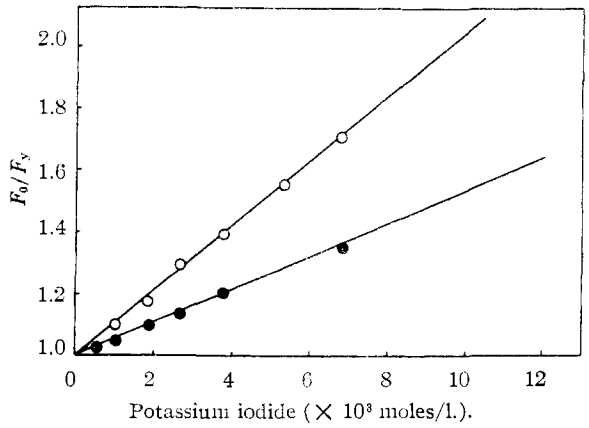

Fig. 5.—Fluorescence quenching by potassium iodide. Dye concentration, 2.3 and 58 micromolar for open circles and closed circles, respectively. F_0/F_y is the fluorescence intensity without quencher relative to that with quencher.

the Stern–Volmer quenching constant was obtained by extrapolation to infinite dilution of dye and is equal to 113 liters/mole. The dependence of the reciprocal of the Stern–Volmer quenching constant upon dye concentration is linear with a slope equal to 186.

The efficiency of quenching by potassium iodide is dependent upon the viscosity of solution. As the glycerol content was varied from 0 to 50%, in a series of solutions each containing 2×10^{-2} *M* potassium iodide, and 1.5×10^{-4} *M* proflavin at *p*H 4, the fluorescence increased by a factor of two. Qualitatively, this variation is what might be expected of a diffusion-controlled quenching process.

3. Fluorescence Quenching by Allylthiourea.—The quenching of fluorescence of proflavin by allylthiourea was performed at three different concentrations of the dye, shown in Fig. 6. Instead of the expected result as found with potassium iodide, the fluorescence quenching increases with higher dye concentrations. Each curve shows a slight positive deviation from a straight line at concentrations of allylthiourea of 10^{-1} *M*. The limiting

(11) P. Pringsheim, "Fluorescence and Phosphorescence," Interscience Publ., Inc., New York, N. Y., 1949, p. 90.

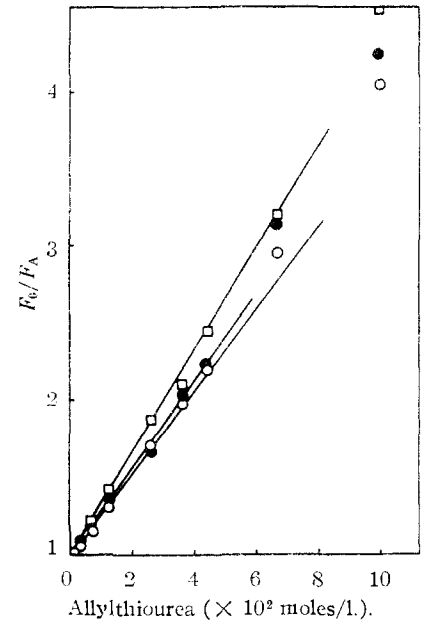

Fig. 6.—Fluorescence quenching by allylthiourea. Dye concentration: 2.3, 12 and 58 micromolar for open circles, closed circles and squares, respectively. F_0/F_A is the fluorescence intensity without quencher relative to that with quencher.

value of this Stern–Volmer quenching constant was obtained by extrapolation to infinite dilution of dye and is equal to 26 liters/mole.

The quenching of fluorescence by allylthiourea is dependent upon viscosity and temperature and is therefore of the diffusional kind. When the solution medium was changed from water to an aqueous glycerol solution of 111 centipoise viscosity allylthiourea became completely ineffectual as a quenching agent up to 10^{-2} *M* concentration. As the temperature of an aqueous solution of 6×10^{-5} *M* proflavin and 6×10^{-2} *M* allylthiourea was raised by 5° increments from 25 to 50° fluorescence decreased 22%. Thus, decrease in the viscosity of medium was paralleled by an increase in the quenching efficiency of allylthiourea. To prevent photoreduction during the process of measuring fluorescence quenching the solutions were always made to include 10^{-5} *M* *m*-phenylenediamine.

D. Phosphorescence.—In glycerol at −90° all the members of class I acridines of Table I are strongly phosphorescent; of class II dyes only compounds 2b and 2c appear so; of class III dyes only compounds 3b (weakly) and 3g (strongly) are phosphorescent.

The phosphorescence intensities under the above conditions was studied as a function of concentration in the case of two dyes. In contrast to acridine orange, with which phosphorescence disappears as its concentration is increased, proflavin shows no such diminution.

Discussion

As seen in Table I, all the acridines having amino substituents in the 3- and 6-position are readily photoreducible under the conditions employed. Interestingly, a similar structural specificity was found by De Bruyn, *et al.*, with regard to the

unique *in vivo* affinity of the aminoacridines for intranuclear nucleoprotein.[12] All of the members of class I show strong persistent phosphorescence in viscous media indicating excitation to a metastable state. On the other hand, the members of class III, with the exception of bis-trypaflavin and to a mild extent 3-aminoacridine, do not show this effect.

Bis-trypaflavin, as well as compound 2e, are the only acridines which do not fluoresce in aqueous solution. In viscous media, however, bis-trypaflavin is strongly fluorescent. Apparently interrotational diffusion processes are suppressed in viscous media.[10] From consideration of its structural relation to members of class I, bis-trypaflavin would be expected to undergo photoreduction in viscous media. This is indeed the case, since a slow photoreduction of the dye is found to take place when the dye is incorporated in a glucose glass containing 10% by weight of allylthiourea.[13]

The other members of class III provide some interesting contrasts. With the exceptions noted above, all fluoresce in water, but do not phosphoresce in viscous media. Monoaminoacridines do not photoreduce, although their reduction potentials as measured by polarography are the same as those of the members of class I[14]; 2,7-diaminoacridine shares in common with the members of class I the feature of symmetry; 9-aminoacridine is atypical with respect to the 9-alkylaminoacridines of class II due to its inability to undergo photoreduction.

Class II is in a median position, showing a poorer photoreductive capacity. The occurrence of luminescence phenomena varies among the members. Compound 2e shows little fluorescence, yet it photoreduces. However, this compound differs from the other acridines in having a potential for being reduced by allylthiourea in the absence of light, where it shows a slow rate after oxygen has been removed. Only two compounds of this class, *i.e.*, 2b and 2c, show phosphorescence to any appreciable extent.

The immediate product of the photoreduction of 3,6-diaminoacridine is undoubtedly the 3,6-diaminoacridan. Oxygen easily reoxidizes the leuco-product of photoreduction, as one would expect from the reduction potential. Spectral evidence of the identity of the product of photoreduction was also obtained. A differential spectrum referred to a solution containing only the reducing agent (allylthiourea, λ_{max} 238 mμ) was taken of a photoreduced solution of proflavin at *p*H 4. The results showed the disappearance of the visible absorption band and the development of a band at about 292 mμ, with an approximate log ϵ of 4.2. Similar constants have been reported to occur for 9,10-dihydroacridine, at 289 mμ, log ϵ 4.14[15,16] and for 3-chloro-7-methoxyacridan, at 292 mμ, log ϵ 4.2,[16] which compounds were prepared by chemical reduction of the corresponding acridines.

The maximum rate of photoreduction of proflavin at *p*H 4 is not accountable in terms of the pK_a's of the dye (namely, 9.7 and *ca.* 1.5). Nor can a maximum be explained by a continuously decreasing reduction potential with decreasing *p*H. The maximum *p*H range does, however, correspond to the pK_a expected of the photoreduction product, which is a substituted homolog of *m*-phenylenediamine. It would also be expected that the retardation be progressively greater with increasing values of *p*H in the region of the pK_a. The effect of retardation of the rates by the product of photoreduction can be demonstrated directly. The effect is also apparent from the fact that for an equivalent concentration of dye an initial rate is greater than an instantaneous rate after a finite period of reaction. There may be another physical basis for maximum activity in this *p*H range, since De Bruyn reports that proflavin, when bound to nucleoprotein, shows maximum fluorescence in the same range.[12]

There are two features of the photochemical properties of proflavin which differ markedly from those of other dyes free in solution. One feature involves the increase of the quantum yield of photoreduction with increasing dye concentration of less than 10^{-5} *M* proflavin (see Fig. 1). In contrast, for example, the fluorescein dyes begin to exhibit a marked decrease in quantum yield with increasing dye concentration in the same concentration range.[3]

The second feature is the strong self-quenching of the fluorescence of proflavin in this same low concentration region (see Fig. 4), whereas, for instance, fluorescein at *p*H 7 does not exhibit this phenomenon except at concentration one hundred-fold greater. Both effects can be interpreted as manifestations of a transition of an electronically excited singlet-state species to a long-lived state, induced by the dye itself. This is expressed in step 3 in the kinetic scheme given below. Step 3 predicts that processes which depend on the concentration of the long-lived species, such as the rate of photoreduction, are related in an inverse manner with the concentration of the fluorescent species. Indeed, certain experiments involving fluorescence serve as a quantitative check upon experiments involving the photoreductive rates.

The over-all features of the kinetics of photoreduction of proflavin are compatible with the scheme

(1) $D + h\nu \rightarrow D^*$ (absorption of light with excitation to the first excited singlet state; the velocity of reaction, V, is proportion to the intensity of the light absorbed, I; $V_1 = k_1 I$).
(2) $D^* \rightarrow D$ (reversion to the ground state by a radiationless transition or by emission of fluorescence; $V_2 = k_2(D^*)$).
(3) $D^* + D \rightarrow D' + D$ (induced transition to the long-lived state; $V_3 = k_3(D^*)(D)$).
(4) $D' \rightarrow D$ (transition of the long-lived state to the ground state; $V_4 = k_4(D')$).
(5) $D' + A \rightarrow$ products (reaction of allylthiourea with the long-lived state to give the leuco-dye; $V_5 = k_5(D')(A)$).
(6) $D' + X \rightarrow D + X$ (quenching of the long-lived state by meta-phenylenediamine; $V_6 = k_6(D')(X)$).
(7) $D^* + Y \rightarrow D + Y$ (fluorescence quenching by potassium iodide; $V_7 = k_7(D^*)(Y)$).
(8) $D^* + A \rightarrow D + A$ (fluorescence quenching by allylthiourea; $V_8 = k_8(D^*)(A)$).

(12) P. P. H. De Bruyn, R. S. Farr, H. Banks and F. W. Morthland, *Exp. Cell Research*, **4**, 174 (1953); F. W. Morthland, P. P. H. De Bruyn and N. H. Smith, *ibid.*, **7**, 201 (1954).
(13) G. Oster, J. Joussot-Dubien and B. Broyde, to be published.
(14) B. Breyer, G. S. Buchanan and H. Duewell, *J. Chem. Soc.*, 360 (1944).
(15) E. R. Blout and R. S. Corley, THIS JOURNAL, **69**, 763 (1947).
(16) A. L. Tarnoky, *Biochem. J.*, **46**, 297 (1950).

Step 3 is prescribed by the dye concentration dependence of the quantum yield of photoreduction and of the fluorescence yield. This step serves as the sole path of generation of the long-lived species. A possible alternative process would be a direct transition from the excited singlet state, $D^* \rightarrow D'$, which has been postulated for other classes of dyes.[3,5] But, this is precluded here by the fact that the quantum yield of photoreduction extrapolates to zero rate at low concentration of proflavin.

A long-lived state of proflavin is involved in the reaction since the rate is retarded by very small amounts of *m*-phenylenediamine. This retarder quenches the phosphorescence in glycerol solution at concentrations at which the fluorescence yield is not affected. From the data shown below the average lifetime of the metastable species is 100,000 longer than the lifetime of the fluorescent species, a fact which allows very small concentration of the inhibitor to be effective. Since other aromatic substances of various chemical structures are similarly effective as retarders, it is most probable that a collisional deactivation process is operating rather than energy transfer over long distances because the latter mechanism requires close structural similarity of the interacting molecules.

Assuming steady-state concentrations for the transient species D* and D′ the dependence of the quantum yield for the initial rate of fading upon the concentration of reactants may be shown to be

$$\Phi = \frac{k_5(\mathrm{A})}{[k_4 + k_5(\mathrm{A}) + k_6(\mathrm{X})]} \frac{k_3(\mathrm{D})}{[k_2 + k_3(\mathrm{D}) + k_7(\mathrm{Y}) + k_8(\mathrm{A})]} \quad (1)$$

The result is represented as a product of two factors. When the rate is studied as a function of D, the first factor can be kept constant, and eq. 1 then corresponds to the empirical dye dependent rate function as given in the Results. Thus, from the dye concentration dependence of Φ, where (X) and (Y) are zero, the limiting value of a/b at zero (A) gives the value of k_3/k_2.

When the rate is studied as a function of (A), the second factor in eq. 3 is effectively constant at low (A). Representing the second factor by k, then eq. 1 corresponds to the rate as a function of (A) as given in the Results. The data of Fig. 2 leads to the value of d/c, and since (X) is zero, this is the value of k_4/k_5.

In the study of retardation with *m*-phenylenediamine, the function R_0/R_x, the ratio of photoreductive rates without (o) and with (x) the retarder, was plotted in Fig. 3 against the concentration of *m*-phenylenediamine (X). Utilizing eq. 1 this function is seen to be

$$R_0/R_x = 1 + \frac{k_6(\mathrm{X})}{k_4 + k_5(\mathrm{A})} \quad (2)$$

If k_6 is assumed to be equal to the value of the theoretical frequency of encounters between diffusing molecules in solution[17] (6.6×10^9 sec.$^{-1}$ liter mole^{-1} in water at room temperature), a value for the sum in the denominator of eq. 2 is obtained which is equal to 5.1×10^4 sec.$^{-1}$. Since we already have the value of k_4/k_5 and since (A) is known, the individual constants can be separated by simultaneous solution. It is found that k_5 equals 1.0×10^6 liters mole^{-1} sec.$^{-1}$ and that k_4, the reciprocal lifetime of D′, equals 2.5×10^2 sec.$^{-1}$. The latter value indicates a rather long but not unreasonable lifetime for the metastable state. When k_5 is compared to k_6, it is realized that less than one in every thousand encounters leads to reduction. If an energy of activation requirement were solely responsible for the low efficiency, a value of 5.5 kcal. mole^{-1} can be calculated for it.

In the same way that k_4 and k_5 could be separated by the information involving the use of a retarder, so can information involving the use of a fluorescence quencher make possible the separation of the constants in the ratio k_3/k_2. Analysis of the quenching data is brought about using the function F_0/F_y, which is equal to $(D^*)_0/(D^*)_y$, where (D*)is the steady-state concentration of the fluorescent species D*. On the basis of the eight-step scheme a Stern–Volmer expression is obtained for the function $F_0/F_y = 1 + k_7(\mathrm{Y})/[k_2 + k_3(\mathrm{D})]$, where (Y) is the concentration of potassium iodide. The limiting Stern–Volmer quenching constant at infinite dilution of dye is given by k_7/k_2, and the dye dependency of the quenching constant is related to k_3/k_7. The complexity in the quenching process is seen to be due to the operation of reaction step 3 of the scheme. If one assumes that k_7 is also equal to the theoretical encounter frequency calculated for k_6, a value of 5.8×10^7 sec.$^{-1}$ is obtained for k_2, and 1.2×10^{12} liters mole^{-1} sec.$^{-1}$ is calculated for k_3. The reciprocal of k_2, equal to 1.7×10^{-8} sec., is the mean fluorescence lifetime and is of common magnitude. The constant k_3 will be discussed below.

It may be stressed at this point that because of the dependence of the quenching constants upon dye concentration, quenching constants, as reported in the literature, have exact meaning only if the concentration of proflavin is qualified or the value has been extrapolated to infinite dilution of proflavin.

The assumption that k_7 may be equal to the theoretical frequency of encounters between diffusing molecules in solution rests upon the general knowledge that iodide ion is a most effective collisional quenching agent. The magnitude of the quenching constant is that expected for collisional process. The chemical dissimilarity between proflavin and iodide ion would perhaps preclude a quenching process due to complexation or energy transfer. Moreover, quenching by iodide is found for our case to be viscosity dependent and is thus diffusion controlled.

The fluorescence quenching by allylthiourea is treated in a similar manner. An expression is obtained for $F_0/F_A = 1 + k_8(\mathrm{A})[k_2 + k_3(\mathrm{D})]$. It is seen that the limiting value of the quenching constant at infinite dilution of dye is k_8/k_2. Employing the value of k_2 obtained previously, a value of 1.5×10^9 liters mole^{-1} sec.$^{-1}$ is derived for the rate constant k_8. Thus allylthiourea has 23% of the quenching efficiency of potassium iodide.

The slight increase of the quenching constant with increasing dye concentration is contrary to that

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predicted and found for potassium iodide. Apparently, at higher concentrations of allylthiourea some supplementary interaction with dye takes place. Besides causing deviation in fluorescence data, the interaction manifests itself in the higher order dependence of k_3/k_2 shown in Fig. 1, and the pronounced deviation from a straight line which is evident in Fig. 2 at high concentration of reductant. Though this high concentration effect due to allylthiourea was not further investigated, the values of the rate constants are unaltered by this effect.

The eight-step scheme is self-consistent. Fluorescence quenching with potassium iodide yields the values of k_2 and k_3. A value of the ratio k_3/k_2 was obtained previously from the dye concentration dependence of the quantum yield of photoreduction. Using the value of k_2 an independent determination of k_3 is obtained. Its value is calculated to be 2.3×10^{12} liters mole^{-1} sec.$^{-1}$ and is twice as large as that obtained above. This latter value is favored, since the previous determination is based on a small amount of data and is less direct. Fortunately, a third independent evaluation of the ratio k_3/k_2 is possible and is derivable from the data of fluorescence, self-quenching, and the function $F_0/F_D = 1 + (D)k_3/k_2$. In the absence of self-quenching, the observed fluorescence intensity is proportional to the dye concentration, as exists in extremely dilute solution, such that $F_0 = K(D)$. Therefore

$$\frac{F_0}{F_D} = \frac{K(D)}{F_D} = \frac{K}{(F/D)_D}$$

The expression of the reciprocal relative fluorescence in Fig. 4 may be thought of as a relative fluorescence normalized per molecule of dye, the value of K being so chosen that the ratio is equal to unity at infinite dilution. The data of several runs under various conditions of temperature and viscosity yields a value of $(3.6 \pm 0.4) \times 10^4$ liters mole^{-1} for k_3/k_2. This value checks extremely well with that obtained from photoreduction and is preferred for the calculation of k_3, which is finally given as 2.1×10^{12} liters mole^{-1} sec.$^{-1}$.

The magnitude of k_3 which describes a bimolecular interaction is of the order which has been found in the study of systems thought to represent energy transfer.[6,18,19] The magnitude of the rate of interaction is over one hundred times as great as that possible in a diffusion controlled reaction, (compare k_3 with k_6). Indeed, the self-quenching of fluorescence is independent of viscosity.

The self-quenching of fluorescence is independent of temperature, which makes improbable the possibility of quenching due to complexation. Furthermore, the absorption spectra over a wide concentration range shows no evidence of complexation at *p*H 4.

A reasonable order of magnitude may be estimated for the rate constant of a bimolecular reaction step involving resonance interaction on theoretical grounds. The rate equation for step 3, $D^* + D \rightarrow D' + D$, may be written as a pseudo-first order reaction since the gross concentration of the dye is essentially constant; thus $2.3 \log (D^*)_0/(D^*) = k_3t(D)$, where the zero subscript refers to the initial concentration. A theoretical value for the time period of transfer of energy has been derived.[20] For the case of exact resonance, involving radiation of wave length of 4700 Å., which value occurs in the region of overlap of the absorption and fluorescence spectral bands of proflavin, the relation is obtained that $t = 6.9 \times 10^7 r^3$ cm.$^{-3}$ sec., where r is the distance of separation of the resonating molecules. If r^3 is equated to $1/[(6 \times 10^{20}(D)]$, and the value of $t(D)$ is substituted into the integrated rate expression, the result, $\log (D^*)_0/(D^*) = k_3(5.0 \times 10^{-14}$ sec. mole liter^{-1}), is obtained. It is concluded that k_3 cannot be much larger than 2×10^{13} if one hopes to observe fluorescence at all; yet, when k_3 is only 10^{12} about 11% fluorescence loss would be expected. The value of k_3 found for proflavin is in this range and, therefore, supports the contention that the phenomenon of energy transfer is operating here.

The induced effect of dye in the ground state upon the excited species is acting over large distances of separation. The specific relative fluorescence yield of proflavin is 50% at a concentration of 2×10^{-5} mole/liter, corresponding to an average intermolecular distance of 470 Å. This is an unusual result compared with theory[20-23] and experimental observation,[24] which find 100 Å. to be an average value for r. A discussion of this discrepancy is considered elsewhere (ref. 1a).

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