FLASH PHOTOLYSIS STUDY OF THE REACTIONS BETWEEN PHOTOEXCITED FLUORESCEIN (AND SIMILAR DYES) AND SELECTED SUBSTRATES WHICH ARE ON THE ONE HAND ELECTRON DONORS AND ON THE OTHER HAND ELECTRON ACCEPTORS

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Abstract

The primary processes occurring in the photo-oxidation and photoreduction of xanthene and acridine dyes in aqueous solutions were studied by means of the flash photolysis technique, and intermediary products appearing in these reactions were characterized.

The triplet state of fluorescein, appearing in the photolysis of the dye in deoxygenated sulfuric acid was studied with respect to absorption spectrum and decay rate. A cationic species of the triplet was identified and its acid strength was determined. The triplet was found to be deactivated by processes analogous to those previously reported for fluorescein in aqueous solution.

The photo-oxidation of fluorescein by oxygen was studied in acidic as well as in alkaline solution. The rate constant for quenching of the triplet state of the dye by oxygen was found to have a value corresponding to a diffusion-controlled reaction. "Chemical" quenching was observed to occur only to a very small extent, producing semioxidized fluorescein, which reacts further with the formation of permanent products.

The photo-oxidation of fluorescein by iron(III) was studied in 0.1 to 10 <u>M</u> sulfuric acid. The spectra of the cationic and neutral forms of semioxidized fluorescein, formed by the reaction between the dye triplet and the metal ion, were recorded and the acid strength of the cationic species determined. Fluorescein was found to be oxidized reversibly

in concentrated sulfuric acid solutions, whereas permanent photoproducts are formed in dilute acid.

On the basis of the results of the study of the reaction between fluorescein and iron(III), the dye photo-sensitized reduction-oxidation reaction between iron(III) and ethylenediamine tetraacetic acid was studied. According to the photosensitization mechanism proposed, the triplet state of the dye reacts first with the oxidizing agent to give semioxidized fluorescein, which in turn is reduced to the original dye by reaction with the EDTA.

The semioxidized form of fluorescein was found to appear also in the photo-oxidation of fluorescein by duroquinone. Durosemiquinone was expected to appear as a transient in the oxidation reaction but no light absorption due to this species could be detected. The reaction between duroquinone and the triplet state of fluorescein was found to be mainly diffusion-controlled.

The influence of the carboxyl group of fluorescein on the photochemical activity of the dye was investigated in a comparative study of the corresponding compound lacking the carboxyl group, that is, 6-hydroxy-phenyl-fluoron. The reversible photoreactions of this dye in deoxygenated aqueous solution were studied, giving as the main result that the coulombic repulsion effect of the ionized carboxyl group decreases the rate of concentration quenching of the triplet state of the dye.

In order to compare the photoactivity of xanthene dyes with that of acridine dyes, a flash photolysis study of proflavine in deoxygenated aqueous solution was undertaken. A transient photoexcited product of the dye was observed, probably representing the proflavine triplet. The absorption spectrum of this metastable product and its decay was studied over a range of acidities.

Main Body of the Report

Xanthene and acridine dyes, such as for example fluorescein, eosin, proflavin, or acridine orange, are known to be efficient photosensitizers of reduction-oxidation reactions. The triplet state of the dye is believed to be responsible for the photosensitizing activity, and the actual transfer of absorbed light energy from the dye to the reactants probably proceeds by a sequence of chemical reactions, which are of a reversible nature as far as the sensitizer is concerned. The subject of the present research program has been to study the mechanisms by which the photoexcited dye acts upon the oxidizing and reducing agents involved in the sensitized reduction-oxidation reactions, and, in particular, to characterize the intermediary products appearing in these processes.

The flash photolysis method is in general very suitable for the study of photochemical reactions, but a very high flash light output and a very short flash duration time proved to be required in order to produce measurable transient absorption changes of the reaction systems of interest. The present research was made possible thanks to the development at this Institute of flash photolysis units of extremely high flash light intensities and short flash duration times (Ref. 1-4).

A series of photochemical studies of xanthene and acridine dyes, in the presence or absence of oxidizing and reducing agents were initiated, and are described in the following sections. A previous study (Ref. 5) of the triplet state of fluorescein in aqueous solution was used as a basis for the present work, and the results of this study are for reference summarized in an introductory section.

The different studies were performed under very similar experimental conditions, and the experimental procedure is therefore described in a separate section.

1. Experimental conditions

The flash photolysis method was used in the studies to be described. The flash photolysis apparatus (Ref. 1) consisted of a pair of 70 cm long, straight discharge lamps and of a capacitor bank of 350 µF, max. voltage 14 kV. Except where otherwise stated, an energy of 6000 J (100 µF, 11 kV) was discharged across the lamps, giving a flash duration time of 60 µsec. The reaction cells, which were 20 and 60 cm long, were provided with jackets filled with a filter liquid consisting of copper sulfate and ferric alum in dilute sulfuric acid. The filter transmitted light of wavelengths 390 to 630 mµ, *i.e.*, only visible light was used for excitation. The filter solution was circulated through a thermostat held at 25° C.

The flash-induced transient changes in light absorption of the reaction system were measured photoelectrically. A xenon lamp (Osram XBO 301) was used as monitoring light source, or when very high stability was required, a zirconium arc lamp (Sylvania, 100 W), fed from an accumulator. After passing through the reaction cell the light was dispersed in a monochromator (Zeiss MM12) and the intensity of the light at the exit slit measured by means of a multiplier phototube (EMI 9552 or 9554, DuMont 6911) and an oscilloscope (Tektronix 533) with photographic recording.

The solutions were thoroughly deoxygenated by an evacuation - argon-saturation procedure repeated several times.

The dyes were purified chromatographically, and other chemicals were reagent grade.

2. Summary of previous studies on the triplet state of fluorescein

Fluorescein occurs in four different protolytic forms in aqueous solution of pH 0 to 13. The following table gives the absorption maxima of these forms in the visible range, as well as their protolysis constants:

cation	abs.max.	437 mμ	pK_a	2.2
neutral molecule		437		4.4
monovalent anion		455, 475		6.7
divalent anion		490		

Excitation of the dye by visible light brings the dye to the first excited singlet state, from which it rapidly returns to the ground state by fluorescence or by a radiationless transition. However, there is also a certain probability of conversion to a metastable state of comparatively long lifetime, presumably the triplet state of the dye.

Using high-intensity flashes complete conversion of the dye to the triplet state was achieved, making possible a study of its absorption spectrum and reaction mechanisms. Only two protolytic forms of the triplet state could be distinguished by their absorption spectra. In acidic solution, the triplet has a diffuse spectrum covering the visible and near infrared, with a maximum at 480 mμ, and in alkaline solution a diffuse spectrum with a maximum at 540 mμ is observed. The 'acid' spectrum probably is due to the neutral and monoanionic triplet forms, which are expected to have identical spectra, while the 'base' spectrum should be due to the dianionic triplet.

The following triplet reactions were observed in deoxygenated aqueous solutions:

$T \longrightarrow S$ (rate constant k_1) (1)

$T + T \longrightarrow S + S$ (k_2) (2)

$T + S \longrightarrow S + S$ (k_3) (3)

$T + T \longrightarrow X + R$ (k_4) (4)

$T + S \longrightarrow X + R$ (k_5) (5)

where S, T, X, and R represent unexcited fluorescein, the triplet, the semioxidized dye, and the semireduced dye, respectively.

In acidic solutions, where the 'acid' triplet spectrum appears, the following rate constants were found:

$k_1 = 600\ \mathrm{sec}^{-1}$, $k_2 + 2k_4 = 3.5 \times 10^9\ \underline{M}^{-1}\ \mathrm{sec}^{-1}$,

and in alkaline solutions, where the 'base' triplet spectrum predominates:

$k_1 = 50\ \mathrm{sec}^{-1}$, $k_2 + 2k_4 = 1.7 \times 10^9\ \underline{M}^{-1}\ \mathrm{sec}^{-1}$.

The semioxidized dye has a strong absorption peak at 428 mμ in solutions of pH 0 - 13 but negligible absorption above 500 mμ. In this pH range the neutral form of the radical probably appears. The semireduced dye has a strong peak at 355 mμ in the pH range 0 - 9, and at 394 mμ in more alkaline solutions. The 355 mμ peak probably is due to the neutral and monoanionic forms of the semireduced radicals, and the 394 mμ peak to the dianionic radical. No absorption was observed in the visible range.

The radicals were found to react rapidly by the reactions

$X + R \longrightarrow S + S$ (k_6) (6)

$X + X \longrightarrow X_2$. ($k_7$) (7)

The dimer of the semioxidized radical, X_2, is probably dissociated to a certain extent, and ultimately all radicals are consumed by reaction (6). The net result of the reactions (1) to (7) is the reappearance of the fluorescein in its ground state.

3. The triplet state of fluorescein in sulfuric acid

The purpose of the present study was to investigate the possibility of the existence of a cationic form of the triplet state of fluorescein, which was expected to appear in solutions of high acidities.

Fluorescein in 0.1 to 10 M sulfuric acid was studied by the flash photolysis method. Flash-exposure of 0.05 to 4 μM solutions of the dye in deoxygenated sulfuric acid produced a complete fading of the absorption peak of the fluorescein cation, which appears in this acid concentration range, and a diffuse spectrum covering the visible and near infrared appeared instead. In sulfuric acid of concentrations greater than 4 M a spectrum with a peak at 650 mμ was observed, while in more dilute acid the spectrum of the neutral dye triplet appeared (see Sect. 2). It was concluded that the 650 mμ peak is due to the cationic dye triplet. The protolysis constant of the triplet cation was determined from measurements of the transient increase in absorption at 650 mμ produced by flash-excitation of fluorescein in sulfuric acid of varying concentration. A pK_a value of -2.2 was obtained, using the Hammett acidity function as acidity scale.

The spectrum of the cationic triplet is very similar to the triplet spectrum of fluorescein in boric acid, measured by Lewis *et al.* (Ref. 6). This result supports the hypothesis made in Sect. 2 that the metastable photoexcited product of fluorescein observed in aqueous solution is indeed the triplet state of the dye.

The decay of the triplet was assumed to proceed according to the reactions (1) to (5). The rate constants of the reactions of the cationic triplet in 9 $\underline{M}$ sulfuric acid were determined from measurements of the rate of decay of the triplet absorption at 650 mμ, after flash-exposure of 0.05 to 2 μ$\underline{M}$ fluorescein solutions. The values

$$k_1 = 220 \text{ sec}^{-1}, \quad k_2 + 2k_4 = 0.8 \times 10^9 \ \underline{M}^{-1} \text{ sec}^{-1}$$

were obtained. The value of the second order rate constant is indication of a diffusion-controlled reaction.

The occurrence of reactions (4) and (5) was shown by the appearance of absorption peaks characteristic of semi-oxidized and semireduced fluorescein (Sects 2 and 5). In sulfuric acid of concentration greater than 2 $\underline{M}$, peaks were observed at 355 and 442 mμ, and in weaker acid at 355 and 428 mμ. The 355 mμ peak is due to the neutral semireduced dye radical, and the 428 mμ peak to the same radical in a neutral form.

The extinction coefficients of the radicals at the absorption peaks are known, and it was thus possible to measure the actual concentration of the radicals. Using the values obtained the ratio between the rate constants of the two competing triplet quenching reactions (2) and (4) were calculated, giving the result:

$$k_2 = k_4.$$

A study of the rate of decay of the triplet cation under conditions of partial conversion of the dye to the triplet state showed that unexcited dye molecules present in the solution did not affect the decay rate. This gave an upper limit of the rate constants of the reactions (3) and (5):

$$k_3 + k_5 < 0.04 \times 10^9 \ \underline{M}^{-1} \ \mathrm{sec}^{-1}.$$

A study of the disappearance of the radicals showed that in sulfuric acid of less than 2 $\underline{M}$ the reactions (6) and (7) occur, while in more concentrated acid the dimerization reaction (7) is not observed.

The reactions of the triplet state of fluorescein in sulfuric acid are thus seen to be very similar to those observed in aqueous solutions, and the present study supports the correctness of the reaction scheme proposed in Sect. 2.

Technical and Scientific Report No. 1 gives a detailed account of the studies presented in this Section.

4. Photo-oxidation of fluorescein by oxygen

The investigation of the reaction mechanisms in dye photosensitized oxidation reactions and of the interaction between oxygen and molecules in the triplet state have been the subject of several studies. In general, oxygen has been found to be a very efficient quencher of the triplet state, a fact that has been explained as being due to the triplet nature of oxygen.

In the present work the reactions between oxygen and the triplet state of fluorescein have been studied by the flash photolysis method. The investigation is based upon the results of the previous study of the triplet state of fluorescein in deoxygenated aqueous solution, described in Sect. 2.

Experimental. The preparation of fluorescein solutions of known oxygen concentrations in the micromolar concentration range presented certain difficulties. The following method was devised, and proved to give reproducible results.

The reaction cell is filled with thoroughly degassed fluorescein solution in the way described previously (Ref. 5). Argon at atmospheric pressure is applied above the solution and the reaction cell is removed from the vacuum line to which it was attached during the filling procedure. The cell neck is closed with a stopper provided with a stopcock, as shown in Fig. 1. An argon stream is swept through the stopcock during this procedure, as indicated in the figure. Argon is also swept around the cell neck to keep air from being introduced into the cell before the stopper has been put on. A known amount of air-saturated water is then introduced through the open stopcock by means of a stainless steel capillary, without interrupting the argon stream. The capillary is removed and the stopcock closed. Conditions are arranged such that the stopcock is completely filled with solution. Thorough mixing was obtained by means of glass balls in the reaction cell. Blank tests showed that with this filling procedure the amount of air accidentally introduced increased the oxygen concentration by less than 0.03 $\mu\underline{M}$, a value calculated by the use of the oxygen quenching constant reported below. The oxygen concentration in the air-saturated water was found to be 2.8×10^{-4} $\underline{M}$.

Other experimental details are described in Sect. 1.

Results and discussion. Flash-excitation of fluorescein in aqueous solution gives as the first long-lived product the dye in the triplet state, which disappears according to the reactions (1) and (5). When the solutions contain oxygen the following two reactions are expected also to take place.

$$T + O_2 \longrightarrow S + O_2 \qquad (k_8) \qquad (8)$$

$$T + O_2 \longrightarrow X + (O_2)_{red} \qquad (k_9) \qquad (9)$$

where $(O_2)_{red}$ represents HO_2 or O_2^-.

Assuming that the dye is converted quantitatively to the triplet state on flashing, the triplet decay immediately after the flash-exposure can be expressed as:

$$-\left[\frac{d(\log_e c_T)}{dt}\right]_0 = k_1 + (k_2 + 2k_4)\ C + (k_8 + k_9)C_{O_2} \qquad /1/$$

where c_T represents the triplet concentration, C the overall dye concentration, and C_{O_2} the oxygen concentration.

The rate constant for triplet quenching by oxygen, $(k_8 + k_9)$, was determined from measurements at oxygen concentrations ranging from 1 to 5 µM. The fluorescein concentration was kept low (0.2 µM) in order to minimize the effect of the concentration quenching reactions (2) and (4). The investigation was carried out at pH 2, where the neutral triplet appears, and at pH 12, where the dianionic triplet is formed. The triplet concentration was determined from measurements at 510 mµ in the acidic solution, and at 540 mµ in the alkaline solution. Fig. 2 shows values of the left hand side expression of Eq. /1/ as a function of the oxygen concentration at pH 12. The slope of the line gives the quenching constant

$$k_8 + k_9 = 1.7 \times 10^9\ \underline{M}^{-1}\ \sec^{-1}.$$

at pH 2 the value

$$k_8 + k_9 = 1.2 \times 10^9\ \underline{M}^{-1}\ \mathrm{sec}^{-1}$$

was found.

Oxygen apparently is a very efficient quencher of the triplet state of fluorescein: the high values of the rate constants are an indication of diffusion-controlled reactions.

The photo-oxidation of fluorescein probably proceeds by the 'chemical' quenching reaction (9), and it is therefore of interest to know the rate constant of this reaction. This constant was determined by measurements of the amount of semioxidized dye formed after flashing. In the absence of oxygen this radical is formed by the reactions (4) and (5), and in the presence of oxygen also by reaction (9). Semioxidized fluorescein has a pronounced absorption peak at 428 mμ in the pH range studied, and its extinction coefficient at the peak is known. From measurements of the absorption changes produced at this wavelength the values

$$k_9 < 2 \times 10^7\ \underline{M}^{-1}\ \mathrm{sec}^{-1} \text{ at pH 2,}$$

and

$$k_9 = 1.2 \times 10^8\ \underline{M}^{-1}\ \mathrm{sec}^{-1} \text{ at pH 12}$$

were obtained. Reaction (9) apparently is not of great importance in the quenching process, and the values obtained indicate that the quantum yield of photo-oxidation of the dye should be low. In addition, there is an indication that a reverse raction between the products of reaction (9) takes place, whereby fluorescein in the ground state is regenerated, and this latter reaction should reduce the quantum yield of photo-oxidation even further. It would be of great interest to determine this quantum yield, for comparison with the value that can be calculated from the results presented in this study.

5. Photo-oxidation of fluorescein by iron(III)

A flash photolysis study of the photo-oxidation of fluorescein in 0.025 M perchloric acid by ferric salts was reported in Ref. (5). The formation of permanent oxidation products was revealed by the color darkening of the flash-exposed dye solutions. The reactions

$$T + Fe(III) \longrightarrow S + Fe(III) \tag{10}$$

$$T + Fe(III) \longrightarrow X + Fe(II) \tag{11}$$

were shown to occur. The semioxidized fluorescein was believed to be the precursor of the permanent photoproduct, which was assumed to be formed by a reaction between two semioxidized radicals:

$$X + X \longrightarrow \text{photoproducts.} \tag{12}$$

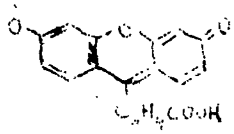
In the present study the photo-oxidation of fluorescein by iron(III) in sulfuric acid has been investigated. In dilute sulfuric acid the reactions suggested above were found to occur. It was possible to obtain 100 per cent yield of semioxidized radicals by flash-excitation of fluorescein in the presence of excess ferric ions, thus allowing a detailed determination of the absorption spectrum of the semioxidized dye. The spectrum shown in Fig. 3 was obtained from measurements of transient absorption changes during flash-excitation of a solution of 0.5 μM fluorescein in 0.1 M sulfuric acid, containing 100 μM ferric ions. Apart from the main absorption band at 428 mμ, less intense bands with maxima at 450 and 405 mμ were observed, as well as a diffuse band of low intensity in the infrared, with a maximum at 800 mμ. At the large excess of ferric ions present, the triplet molecules formed by flash-excitation are semioxidized according to reaction (11) within the resolution time of the flash photolysis apparatus.

The semioxidized radicals were found to disappear by a second-order reaction, resulting in the formation of permanent photoproducts to an extent of approximately 90 per cent. The high percentage of change precludes the occurrence of the otherwise plausible disproportionation mechanism

$$X + X \longrightarrow S + \text{photoproducts.} \qquad (12)$$

The reactions leading to permanent products cannot be revealed until the nature of these products is known, and an analysis of the oxidation product is therefore now being undertaken.

It was suggested in Ref. (5) that the 428 mμ peak is characteristic of the neutral form of the semioxidized dye (I). This radical would be expected to accept a proton at high acidities, giving the cationic semioxidized dye (II).



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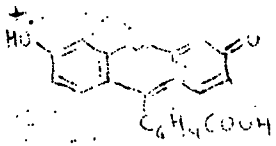

II

Evidence for the appearance of the latter radical ion was obtained by studying the photo-oxidation of fluorescein in concentrated sulfuric acid solutions. From measurements similar to those giving the spectrum of the neutral radical, the spectrum of the cationic radical in 9 M sulfuric acid was obtained, shown in Fig. 3. This spectrum has a pronounced peak at 442 mμ, indication of a band at 410 mμ, and a diffuse, weak band with a maximum at 770 mμ.

The pK_a value of the cationic radical was determined from measurements of the transient increase in absorption at 770 mµ produced by flash-excitation of 0.5 µM fluorescein in sulfuric acid of varying concentrations, also containing 100 µM ferric ions. The value

$$pK_a = -1.5$$

was obtained.

A pronounced difference in reactivity of the cationic radical as compared to that of the neutral radical was observed. Whereas the neutral radical reacts to give permanent photoproducts, the photo-oxidation in strong sulfuric acid was found to be reversible. Although the dye was converted quantitatively to the semioxidized form, a permanent change of only 1 per cent per flash was observed. This result suggests the occurrence at high acidities of the reaction

$$X + Fe(II) \longrightarrow S + Fe(III). \qquad (13)$$

A few experiments were performed with the dye in 9 M sulfuric acid containing iron(II) as well as iron(III). The disappearance of the cationic radical was found to be strongly accelerated by the presence of iron(II), even at low concentrations of the iron(II). This result strongly supports the occurrence of reaction (13).

The reversibility of the photo-oxidation of fluorescein at high acidities may not necessarily be due to a high efficiency of reaction (13) as compared to the reactivity at lower acidities, but may also be explained by a lower efficiency of the dimerisation reaction (7). There is strong indication that the dimeric semioxidized dye is an intermediary product in the process of oxidation of the dye. In Sect. 3 evidence is presented against the occurrence of a dimerization reaction in concentrated sulfuric acid solutions, and this observation tends to support this hypothesis.

6. Photo-oxidation of fluorescein by duroquinone

There is strong indication of the existence of a semi-oxidized form of fluorescein, produced as an intermediate in the photo-oxidation of fluorescein by oxygen (Sect. 4) and by Iron(III) (Sect. 5). The same species also appear to be of importance in photooxidation reactions sensitized by fluorescein.

In order to obtain further information about the semi-oxidized fluorescein radical, a study of the photo-oxidation of fluorescein by duroquinone was undertaken. Duroquinone is known to be readily reduced to the semiquinone radical, and the following reaction with the triplet state of fluorescein therefore would be expected to occur:

$$T + DQ \longrightarrow X + DSQ, \qquad (k_{19}) \quad (19)$$

where DQ and DSQ represent duroquinone and durosemiquinone, respectively. The absorption spectrum of durosemiquinone has been studied by Bridge and Porter (Ref. 7), who found strong peaks due to this species in the wavelength range 400-450 mμ. These authors did not measure the extinction coefficients of the semiquinone at the peaks but suggest a value of 10,000 as the probably order of magnitude.

In order to study reaction (19), aqueous solutions of fluorescein and duroquinone were flash-exposed and the transient changes in spectra measured. Duroquinone was found to be a very efficient quencher of the triplet state. In addition to the chemical quenching reaction (19), physical quenching might possibly occur:

$$T + DQ \longrightarrow S + DQ. \qquad (k_{20}) \qquad (20)$$

The quenching rate constant was obtained from studies of the decay of the triplet spectrum in the presence of duroquinone:

$$k_{19} + k_{20} = 1 \times 10^{9} \ \underline{M}^{-1} \ \sec^{-1}.$$

When excess duroquinone was present the triplet state was not observed, but instead a pronounced absorption peak appeared at 428 mμ, characteristic of semioxidized fluorescein. A quantitative conversion of the dye to this species was observed, indicating that the chemical quenching reaction (19) is more important than the physical quenching reaction (20).

If the semioxidized fluorescein is formed by reaction (19) the absorption peaks of durosemiquinone should show up. However, there was no evidence for the appearance of peaks in the wavelength region 400-450 mμ other than that of the semioxidized fluorescein, and this result sets an upper limit of approximately 1000 for the extinction coefficient of durosemiquinone.

The semioxidized fluorescein disappeared comparatively slowly, by a reaction regenerating the dye in the ground state, probably according to:

$$X + DSQ \longrightarrow S + DQ \qquad (21)$$

The photoreactions were not quantitatively reversible, however, a small amount of permanent products being formed.

Duroquinone has a weak absorption in the visible, and the direct photo-excitation of the quinone could not be avoided. Transient photoproducts of the quinone appeared to a certain extent as a result of this direct excitation, and these interfered in the measurements of the reactions produced by photo-excitation of the fluorescein. A more detailed study is required in order to allow a separation of these two excitation processes from each other.

7. Fluorescein photosensitized oxidation of ethylenediamine tetraacetic acid by iron(III)

In the present study the mechanism of sensitization in the photosensitized oxidation of EDTA by iron(III) using fluorescein as a sensitizer has been studied. Solutions containing: 1) the sensitizer alone, 2) the sensitizer and EDTA, and 3) the complete reduction-oxidation system, were studied by means of the flash photolysis method. It proved possible, by comparison of the kinetics of the photochemical reactions taking place, under these different conditions, to get a detailed picture of the mechanism of photosensitization.

A previous study of this reaction system has been reported by Oster and Oster (Ref. 8). These authors concluded that the photoexcited dye, presumably in the triplet state, reacts with EDTA giving semireduced dye as well as oxidation products of the EDTA. The semireduced dye is then oxidized to its original state by the reaction with the Fe(III)-EDTA complex present in the solution, and the iron(III) is reduced to iron(II) in this process. The study was performed at pH 5.5, and to make comparison with the data presented by these authors possible, the present study was performed at the same pH.

1) Fluorescein in aqueous solution of pH 5.5. The behavior of flash-excited fluorescein in deoxygenated aqueous solution is described in Ref. (5) (see Sect. 2). The triplet state of the dye is formed, and decays by the reactions (1)-(5). Fig. 4 shows the initial rate of decay of the triplet observed after flash-excitation of a 0.1 μM fluorescein solution of

pH 5.5.

The semireduced fluorescein has an absorption peak at 355 mμ at pH 5.5, and the semioxidized form a peak at 428 mμ. Figs 5 and 6 illustrate the formation of these radicals as a result of the electron transfer reactions (4) and (5).

2) Fluorescein in aqueous solution of pH 5.5, containing 1.0×10^{-3} M EDTA. The decay of the triplet formed by flash excitation is accelerated in the presence of EDTA, due to the reaction

$$T + EDTA \longrightarrow R + (EDTA)_{ox} \qquad (k_{22}) \quad (22)$$

where $(EDTA)_{ox}$ represents the product or products of a one--electron oxidation of EDTA. Fig. 4 shows that EDTA at a concentration of 0.001 M enhances the initial rate of decay of the triplet from 1000 sec^{-1} to 1150 sec^{-1}. This gives

$$k_{22} = 1.5 \times 10^5\ M^{-1}\ sec^{-1}.$$

Considering the increase in maximum extinction change at 355 mμ, illustrated in Fig. 5, it is evident that the increased rate is due to chemical quenching by EDTA.

Fig. 6 shows the absorption change as a function time at 428 mμ, where semioxidized fluorescein has a pronounced peak. There is no evidence fot the formation of the semioxidized radical in this case, probably because it is consumed rapidly by the reaction

$$X + EDTA \longrightarrow S + (EDTA)_{ox}. \qquad (23)$$

3) Fluorescein in aqueous solution at pH 5.5, containing 1.0×10^{-3} M EDTA and Fe(III)-EDTA chelate in concentrations up to 10^{-5} M. The decay of the triplet was found to be strongly accelerated in the presence of Fe(III)-EDTA complex, due to the

reactions:

$$T + Fe(III)EDTA \longrightarrow S + Fe(III)EDTA \qquad (k_{24}) \quad (24)$$

$$F + Fe(III)EDTA \longrightarrow X + Fe(II)EDTA \qquad (k_{25}) \quad (25)$$

The quenching constant of the complex can be calculated from the dependence of the triplet decay rate on the concentration of the complex, data being shown in Fig. 4.

One obtains

$$k_{24} + k_{25} = 0.8 \times 10^9 \ \underline{M}^{-1} \ sec^{-1}.$$

A comparison of this value with the triplet quenching constant of Fe(III) (at pH 2), which has a value of 1×10^9 $\underline{M}^{-1}$ sec^{-1} according to Ref. (5), shows that the quenching activity of the iron(III) is not significantly influenced by chelation in this case.

The semireduced fluorescein, formed by the reactions (4) and (5), as well as by the reaction (6) between the triplet and free EDTA present in excess, is rapidly consumed in the presence of Fe(III)EDTA complex, as illustrated by Fig. 5, apparently by the reaction

$$R + Fe(III)EDTA \longrightarrow S + Fe(II)EDTA.$$

The relative importance of reactions (24) and (25) has not been studied in detail. The pronounced increase in absorption at 428 mμ in the presence of the complex, as seen from Fig. 6, indicates that the chemical quenching reaction (25) is the more important.

The above results suggest that the first step in the photosensitized oxidation of EDTA by iron(III) in the presence of fluorescein is the reaction (25) producing semioxidized fluorescein which is reduced to fluorescein by reaction with excess EDTA present (reaction (23)).

The subsequent reactions leading to stable products have not been studied, since they are of no importance in connection with the mechanism of photosensitization.

The study was complicated by the fact that the oxidation products of the EDTA seemed to be efficient quenchers of the triplet state of fluorescein. This showed up as an increase in the rate of decay of the triplet with the number of flashes. This quenching reaction has not been taken into account in the preceding discussion but it is not believed that the general conclusions would be affected by the occurrence of this reaction.

6. A flash photolysis study of 6-hydroxy-9-phenylfluoron.

6-Hydroxy-9-phenylfluoron (HPhF) is identical with fluorescein except for the absence of the carboxyl group. A study of this compound was undertaken in order to investigate the effect of the carboxyl group on the absorption spectrum and decay rate constants of the triplet state of fluorescein.

The fluorescein molecule can be divided into two parts, the xanthene and the phenyl groups, between which there is negligible pi electron interaction. The presence of substituents on the phenyl group therefore would not, to a first approximation, be expected to affect the color of the dye. In alkaline solution the visible absorption spectrum of HPhF is in fact very similar to that of fluorescein: the absorption peak is shifted from 490 to 491 mμ while the peak absorption intensity remains the same. There is the important difference between these two compounds, however, that neutral fluorescein can appear in an uncolored lactonic form, which cannot be realized in the case of HPhF. The protolytic behavior is also affected by the carboxyl substituent.

The protolytic stages of HPhF were determined by measurements of the absorption spectrum of the dye in the pH range 0-13. The dye occurs as a cation at high acidities ($pK_a = 3.2$), while the neutral molecule predominates at lower acidities ($pK_a = 6.3$) and the monovalent anion in alkaline solutions. The corresponding behavior for fluorescein is described in Sect. 2.

A study of the absorption spectrum of the triplet state of HPhF as a function of pH showed that two protolytic species of the dye appear in the pH range 0 - 13, namely the neutral triplet molecule and the monoanionic triplet. The spectrum of the neutral triplet was very similar to that of the neutral fluorescein triplet, while the spectrum of the monoanionic triplet was found to have the same spectrum as the dianionic fluorescein triplet.

The fluorescein triplet dianion and the HPhF triplet monoanion are identical to each other except for the existence of a negatively charged carboxyl group in the former species. This difference in electronegativity was expected to affect the rate constants of the concentration quenching reactions (2) and (4), because of electrostatic repulsion effects. However, a determination at pH 12 of the constant ($k_2 + 2k_4$) for HPhF showed that this constant had the same value as that for fluorescein, within experimental error. Apparently, for a reaction between two triplet fluorescein molecules to take place, the relative orientation required is such that the carboxyl ion repulsion is of no importance. The cationic triplets of HPhF and fluorescein were expected to have very similar rate constants of triplet decay, and a study performed at pH 2 showed this to be the case.

The general conclusion of the study is that the carboxyl group of fluorescein is of no importance as far as the behavior of the triplet state of the dye is concerned.

9. A flash photolysis study of proflavin

The study reported in Sect. (2) of the triplet state of fluorescein revealed the occurrence of the electron dismutation reactions (4) and (5), producing semioxidized and semireduced fluorescein. A similar reaction might possibly occur in solutions of other dyes, that can form stable semioxidized and semireduced radicals. The present study was undertaken in order to study this possibility in the case of the acridine derivative proflavin.

Experimental conditions. Aqueous solutions of 0.1 to 1 μM proflavin were studied by the flash photolysis method, at pH 5 and 11. At the lower pH the cationic form of proflavin appears, having an absorption peak at 445 mμ (extinction coefficient 39000) and at the higher pH the base is formed, with an absorption peak at 395 mμ (ext. coeff. 18,400).

A 0.01 M phthalate buffer was used to establish a pH of 5, and 0.001 M NaOH to give a pH of 11. The solutions were thoroughly deoxygenated.

Results. Under the experimental conditions described above a transient fading of the proflavin spectrum was observed during flashing, and a broad absorption band in the visible and near infrared appeared. The dye was rapidly regenerated to its original state, except for a certain permanent photodegradation of the dye. This change amounted to 8 per cent per flash at pH 11 and to 3 per cent at pH 5.

An increase in light output of the flash did not increase the transient absorption change, showing that the solutions were light-saturated during the flash-exposure. In Fig. 7 the spectrum of the metastable state of proflavin, formed during the flash, is shown. In analogy with the case of fluorescein the metastable state is assumed to be the triplet state of proflavin. The spectra at pH 5 and pH 11 are different from each other in the visible and infrared wavelength range, but have a common absorption peak at 380-390 mμ. It is possible that this peak is not due to the triplet state but represents a radical form of the dye, preceding the appearance of the permanent photoproducts.

If it is assumed that the triplet disappears by the reactions (1) - (5), the rate of decay immediately after flashing can be written as

$$- \left[\frac{d \log_e c_T}{dt}\right]_o = k_1 + (k_2 + 2k_4)\ C \qquad /2/$$

From measurements of the triplet decay at different concentrations, the following values of the rate constants were obtained from plots of the left hand side expression of Eq. /2/ as a function of the dye concentration,

at pH 5: k_1=780 sec^{-1}, $k_2+sk_4=2.1\times10^9$ $\underline{M}^{-1}$ sec^{-1}

at pH 11: k_1=1400 sec^{-1}, $k_2+2k_4 = 1.2\times10^9$ $\underline{M}^{-1}$ sec^{-1}.

The results are very similar to those obtained under similar conditions for fluorescein. There was also some evidence for the occurrence of the electron dismutation reactions (4) and (5) but further work is required to confirm this.

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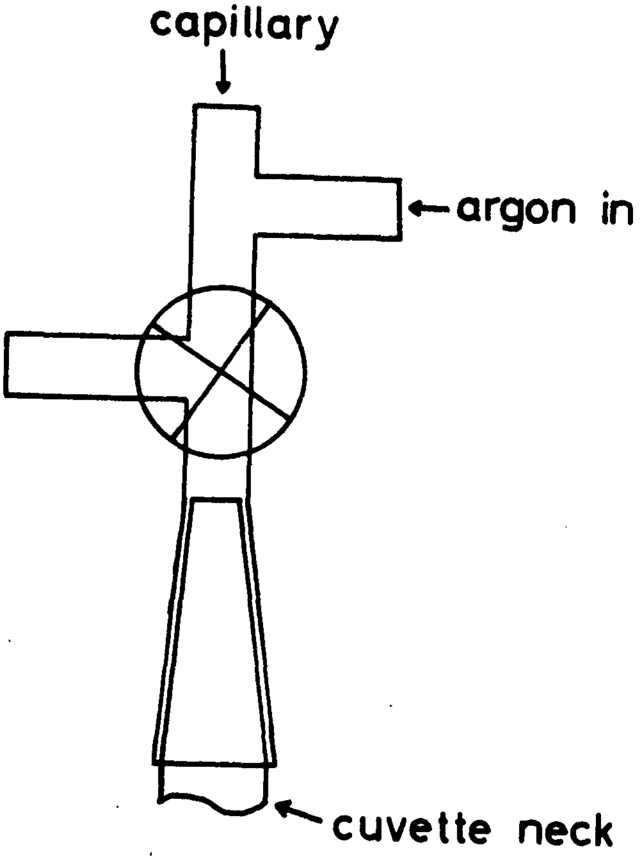

Fig. 1. Cap and stopcock connected to cuvette neck, used for introducing oxygen-containing solutions into the cuvette.

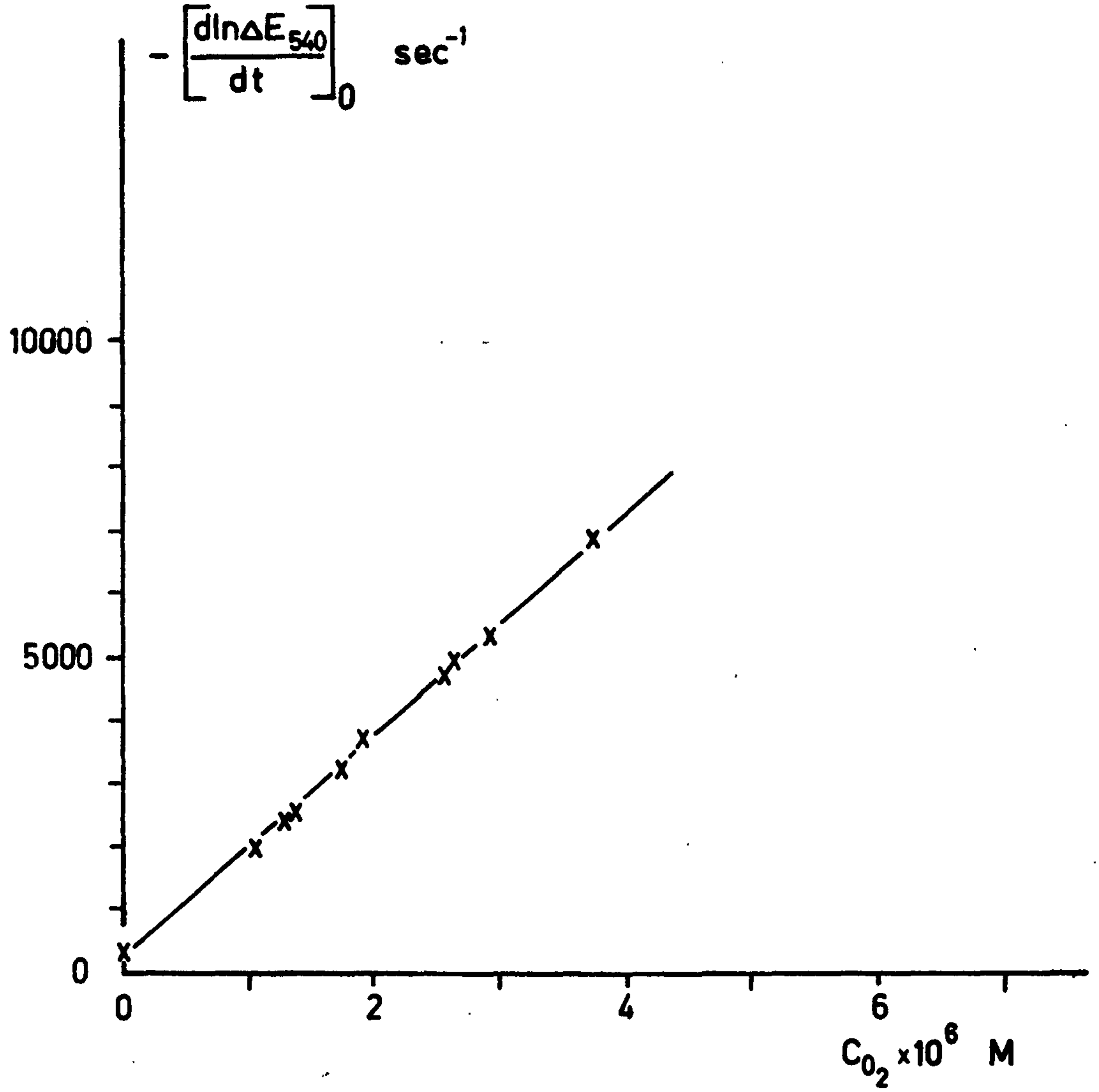

Fig. 2. Initial rate of decay of the triplet state of fluorescein after flash excitation of 0.2 μM fluorescein in aqueous solution at pH 12 plotted against oxygen concentration.

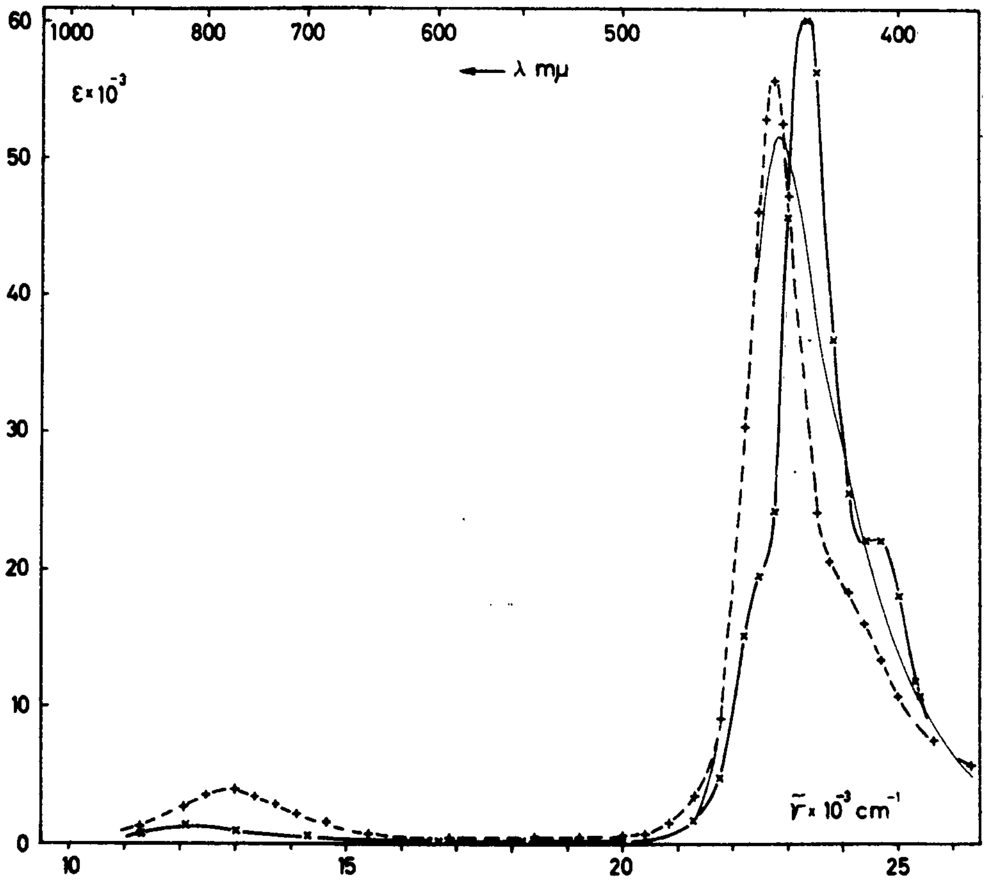

Fig. 3. Absorption spectrum of semioxidized fluorescein as obtained from extinction changes during flash excitation of 0.5 μM in 0.1 M (-x-x-) and 9.3 M (-+-+-) sulfuric acid containing ferric ions at a concentration of 100 μM. The absorption spectrum before flashing is also shown (——).

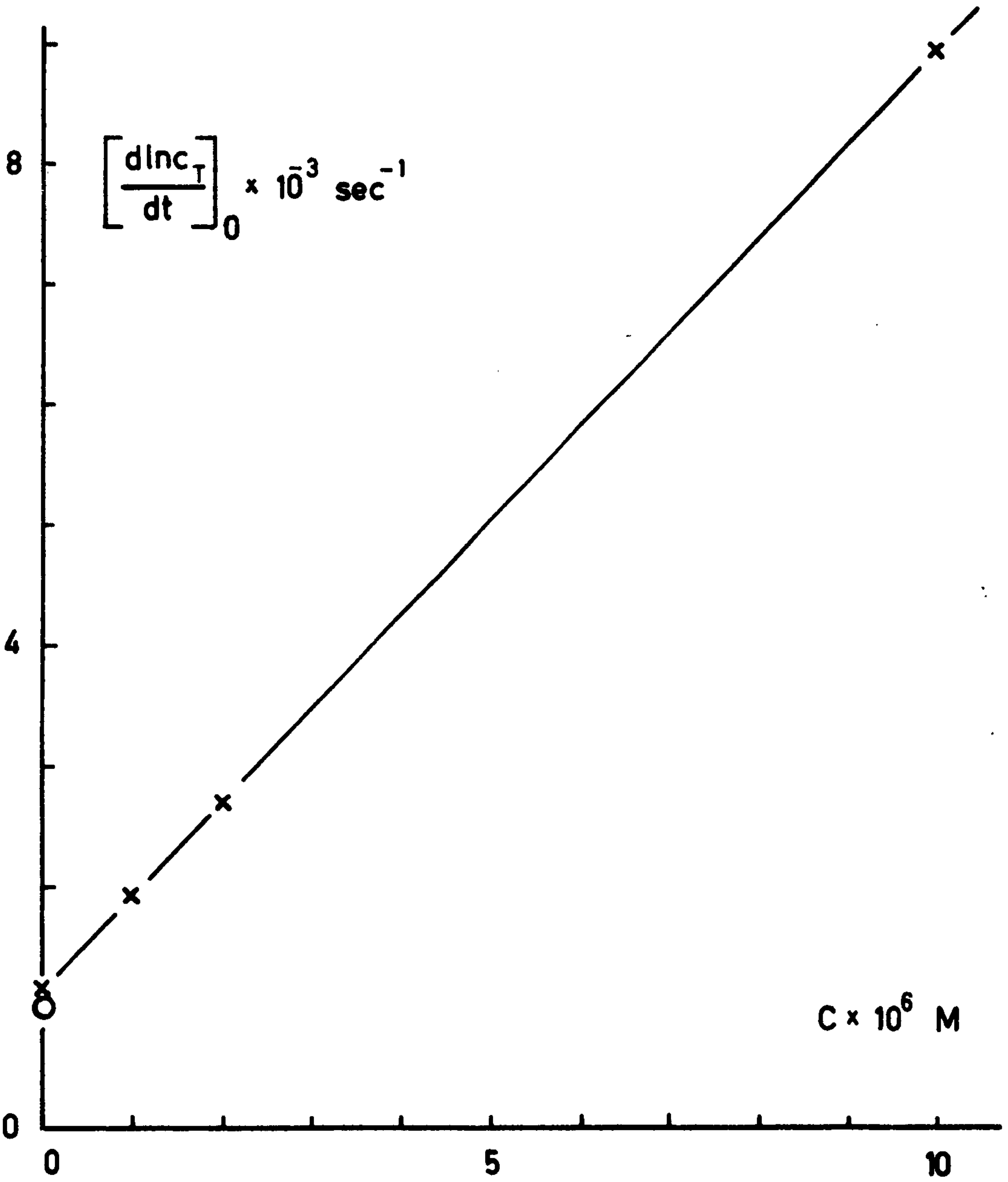

Fig. 4. Initial rate of decay of the triplet state of fluorescein after flash excitation of 0.1 μ$\underline{M}$ fluorescein in aqueous solution at pH 5.5, plotted against the concentration of Fe(III)-EDTA complex in the presence of excess EDTA (10^{-3} $\underline{M}$) (×). For comparison the corresponding rate for a pure fluorescein solution is reproduced (o). Cuvette length 60 cm.

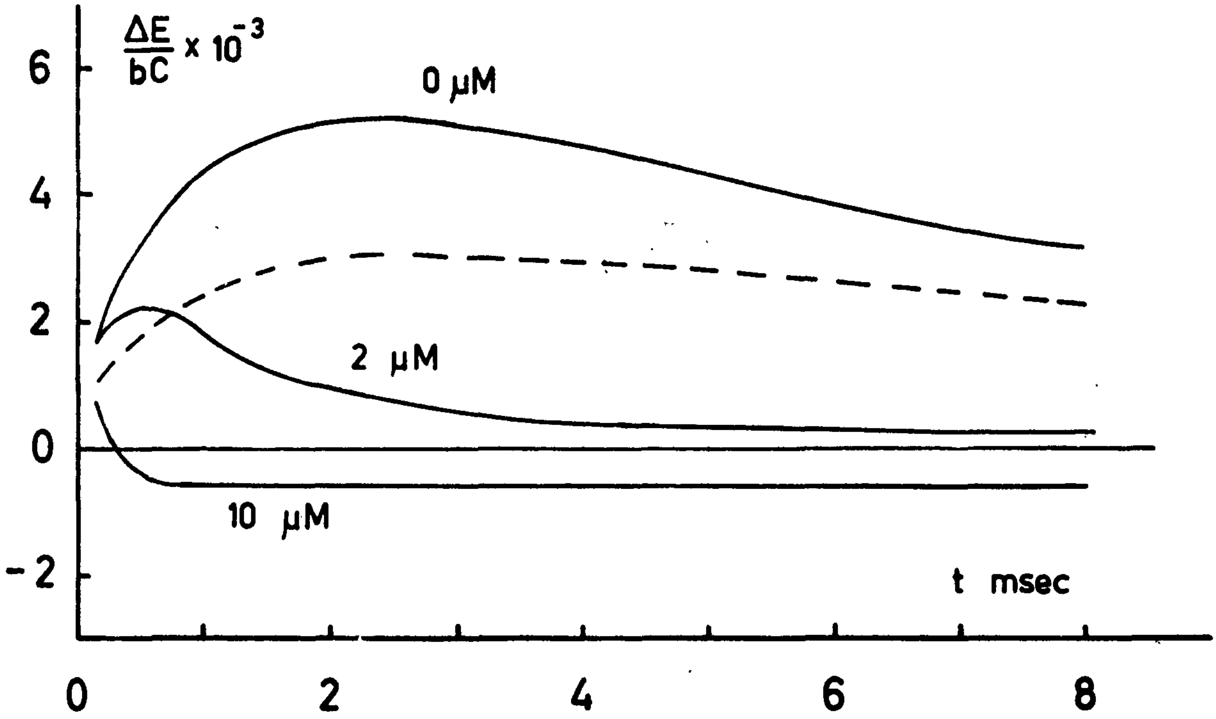

Fig. 5. Extinction change at 355 mμ after flash excitation of 0.1 μM fluorescein in aqueous solution at pH 5.5 ---- . In the presence of Fe(III)-EDTA complex (conc. as indicated in the figure) and excess EDTA (10^{-3} M) —— . Cuvette length 60 cm.

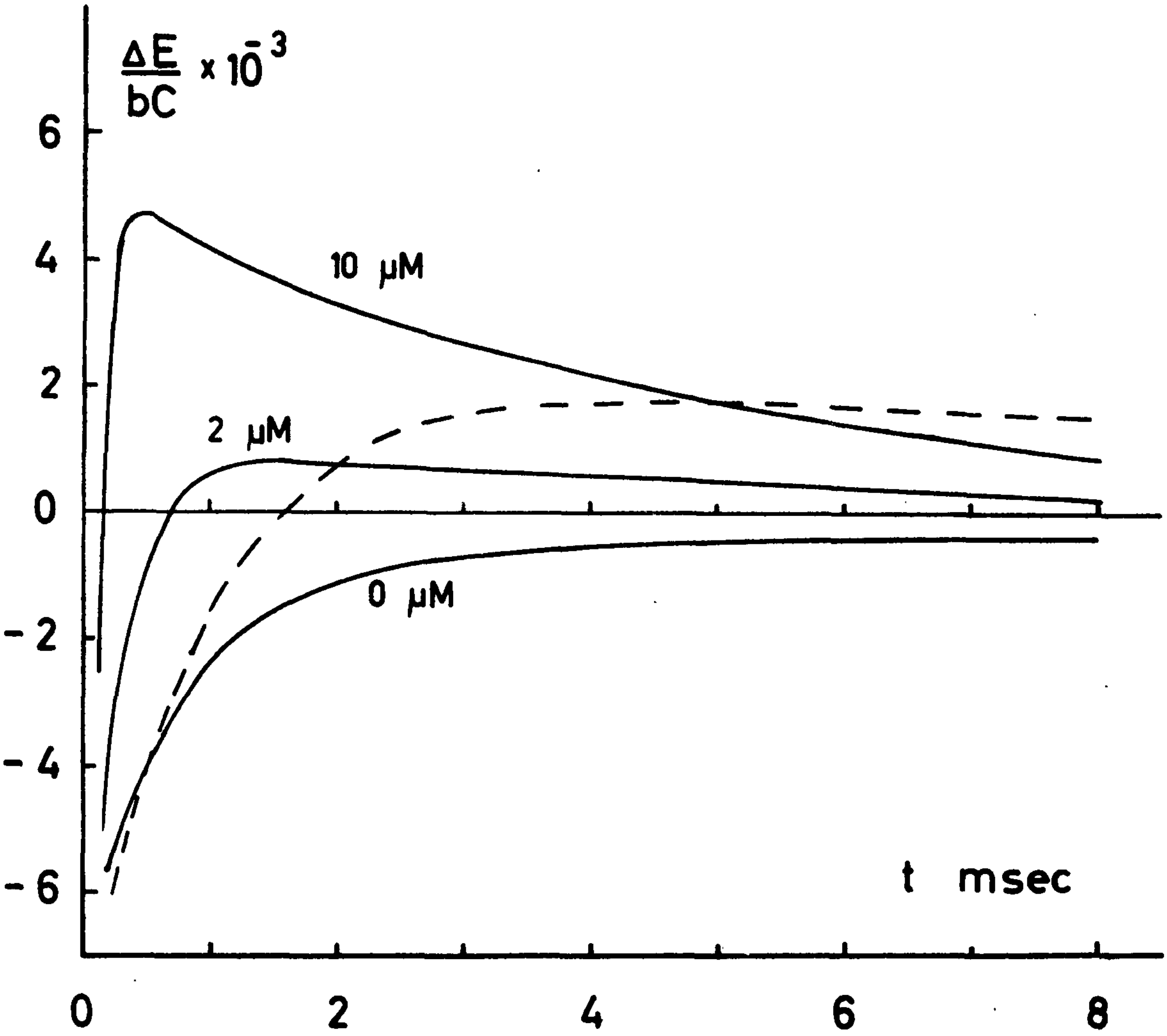

Fig. 6. Extinction change at 428 mμ after flash excitation of 0.1 μM fluorescein in aqueous solution at pH 5.5 ---- . In the presence of Fe(III)-EDTA complex (conc. as indicated in the figure) and excess EDTA (10^{-3} M) —— . Cuvette length 60 cm.

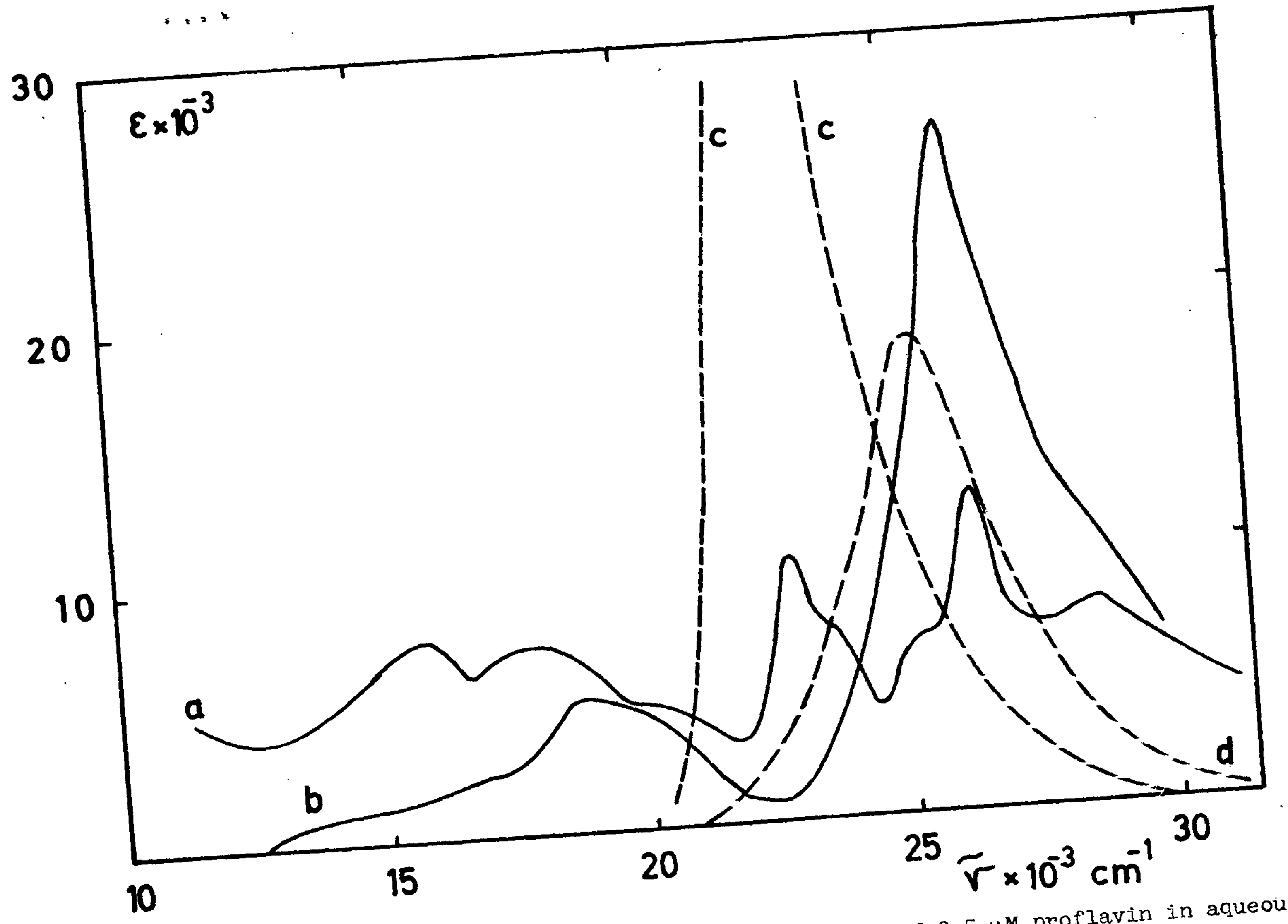

Fig. 7. Transient absorption spectra of flash-excited solutions of 0.5 μ<u>M</u> proflavin in aqueous solutions, <u>a</u> at pH 5, <u>b</u> at pH 11. For comparison the absorption spectra of the dye in the ground state are also shown, <u>c</u> at pH 5, <u>d</u> at pH 11.