Influence of the Triplet Excited State on the Photobleaching Kinetics of Fluorescein in Microscopy

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ABSTRACT The investigation in this report aimed at providing photophysical evidence that the long-lived triplet excited state plays an important role in the non-single-exponential photobleaching kinetics of fluorescein in microscopy. Experiments demonstrated that a thiol-containing reducing agent, mercaptoethylamine (MEA or cysteamine), was the most effective, among other commonly known radical quenchers or singlet oxygen scavengers, in suppressing photobleaching of fluorescein while not reducing the fluorescence quantum yield. The protective effect against photobleaching of fluorescein in the bound state was also found in microscopy. The antibleaching effect of MEA led to a series of experiments using time-delayed fluorescence spectroscopy and nanosecond laser flash photolysis. The combined results showed that MEA directly quenched the triplet excited state and the semioxidized radical form of fluorescein without affecting the singlet excited state. The triplet lifetime of fluorescein was reduced upon adding MEA. It demonstrated that photobleaching of fluorescein in microscopy is related to the accumulation of the long-lived triplet excited state of fluorescein and that by quenching the triplet excited state and the semioxidized form of fluorescein to restore the dye molecules to the singlet ground state, photobleaching can be reduced.

INTRODUCTION

Photobleaching of fluorophores is a phenomenon inherent in fluorescence microscopy. Although photobleaching has its complicating effects, it has been successfully utilized as a parameter in many fluorescence measurement techniques since the 1970s (Peters et al., 1974; Axelrod et al., 1976; Koppel et al., 1986; Jovin and Arndt-Jovin, 1989). In our previous report (Song et al., 1995) we presented experimental observations that demonstrated the non-single-exponential photobleaching behavior. We were then able to explain this observation by using theoretical analysis incorporating photochemical and photophysical properties of fluorescein. We demonstrated that deviation from a single-exponential function was caused by the proximity-induced triplet-triplet and triplet-ground state dye (D-D) reactions. The objective of the study reported here was to provide direct photophysical evidence that the accumulation of the long-lived triplet excited state of fluorescein plays an important role in photobleaching in microscopy.

As early as the 1940s, the light emission by fluorescein (in boric acid "glass") was shown by Lewis et al. (1941) to arise from two processes, which are now called thermally activated delayed fluorescence and phosphorescence (Fig. 1). This phosphorescent state was later identified to be the metastable lowest triplet state (Lewis and Kasha, 1944). Subsequently, different research groups determined the fraction ($\Phi_{S^* \rightarrow T^*}$) of the lowest excited singlet fluorescein molecules (S*) that undergo transition to the lowest triplet excited state (T*). The value of $\Phi_{S^* \rightarrow T^*}$ was experimentally derived to be 0.02 by Adelman and Oster (Oster and Adelman, 1956; Adelman and Lewis, 1956), 0.03 by Gollnick and Schenck (1964), 0.05 by Bowers and Porter (1967), 0.021 by Nemoto et al. (1969), 0.032 by Soep et al. (1972), and 0.03 by Gandin et al. (1983) under various experimental conditions. Although these values represent a very small fraction of the total molecular population of the singlet excited state, the long lifetime of the lowest triplet excited state, compared to that of the singlet excited state, provides favorable conditions for triplet-triplet and triplet-ground state reactions, which in turn lead to the formation of semioxidized and semireduced radical forms (Fig. 2) of fluorescein (Lindqvist, 1960; Kasche and Lindqvist, 1964; see Koizumi and Usui, 1972, for a comprehensive review of their work between 1955 and 1972; Krüger and Memming, 1974). These transient species could lead in part and in multiple steps to nonfluorescing photo-products (Lindqvist, 1960; Kasche and Lindqvist, 1964). The triplet state and the radical forms of fluorescein were shown to be reversibly or irreversibly reduced when reducing agents such as allylthiourea, EDTA, or *p*-phenylene-diamine were added to the fluorescein solution (Oster and Adelman, 1956; Adelman and Lewis, 1956; Lindqvist, 1960; Ohno et al., 1966; Usui and Koizumi, 1967; Grossweiner and Kepka, 1972). At the same time, fluorescence (i.e., the radiative transition from the singlet excited to the ground state) was also quenched to varying degrees at various concentrations of the reducing agents.

The study reported here focused on one thiol-containing reducing agent, mercaptoethylamine (MEA or cysteamine). The original interest in MEA arose from the study by Sheetz and Koppel (1979), who demonstrated that there was a

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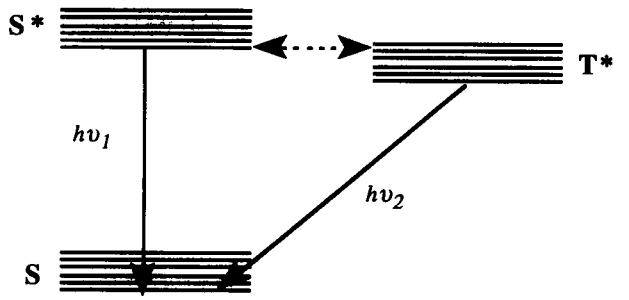
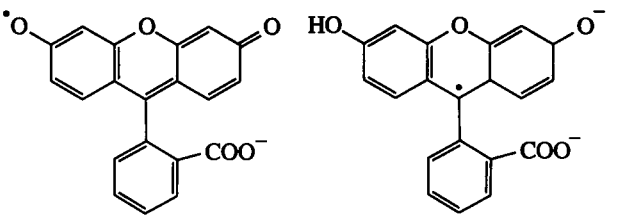


FIGURE 1 The simplified Jablonski energy diagram showing only the downward transitions of luminescence of a generic fluorochrome. A molecule in the lowest singlet excited state (S*) can make a radiative transition back to the ground state (S) and emit fluorescence ($h\nu_1$) on the time scale of nanoseconds (10^{-9} s). It can also make a radiationless intersystem crossing to the lowest triplet excited state (T*). From T* there are two possible radiative pathways that return to the ground state. A triplet state molecule can return to the ground state directly and emit phosphorescence ($h\nu_2$) on the time scale of microseconds (10^{-6} s) to seconds. It can also be thermally activated, cross back to the singlet excited state, and return to the ground state, emitting delayed fluorescence ($h\nu_1$) on the time scale similar to that of the phosphorescence. Emission of fluorescence and thermally activated delayed fluorescence are of the same wavelength, whereas phosphorescence appears at a longer wavelength.



semi-oxidized (X) fluorescein

protonated semi-reduced (R) fluorescein

FIGURE 2 Chemical structures of semioxidized (X) and protonated semireduced (R) radical forms of fluorescein. X absorbs maximally at 428 nm and protonated R at 355 nm in aqueous solution at weakly alkaline pH (Lindqvist, 1960; Krüger and Memming, 1974).

correlation between fluorescein photobleaching and the formation of protein cross-linking, and that applying MEA (or reduced glutathione) drastically reduced protein cross-linking. The exact photophysical mechanism through which MEA reduced photobleaching and protein cross-linking was not known. Our interest in MEA was further stimulated by our own preliminary experiment (see below), in which MEA completely inhibited photobleaching of free fluorescein in solution and significantly reduced photobleaching of bound fluorescein in microscopy without reducing the fluorescence quantum yield.

The objective of the current study was to provide direct photophysical evidence, in support of our previous theoretical analysis (Song et al., 1995), that the accumulation of the long-lived triplet excited-state fluorescein played an important role in photobleaching in microscopy. A better understanding of the action of MEA could help provide this proof. We therefore sought to test the hypothesis that reducing the triplet lifetime to minimize the net loss of ground-state molecules through the long-lived triplet excited state can lead to a decrease in photobleaching in microscopy. The study consisted of four experiments, which successively showed that 1) among other commonly known radical quenchers or singlet oxygen scavengers, MEA was the most effective in reducing photobleaching of fluorescein in solution while not quenching fluorescence; 2) the protective effect of MEA was also shown in a microscopy experiment; 3) the protection by MEA was mediated by reacting with the triplet excited state of the dyes. Eosin, a derivative of fluorescein, was used in this test because of its high $\Phi_{S^* \rightarrow T^*}$ and strong phosphorescence; and 4) the lifetimes of the triplet excited state and semioxidized radical of fluorescein were reduced upon adding MEA and detected by means of direct measurement of the transient absorption change of these populations in the nanosecond laser flash photolysis experiments. Together, these results led to the conclusion that the long-lived triplet excited state of fluorescein plays an important role in photobleaching in microscopy.

MATERIALS AND METHODS

Reagents

All reagents used in this study were of the highest purity available and used without further purification: the free acid form of fluorescein (99% pure, laser grade; Kodak Laboratory Chemicals, Rochester, NY); the free acid form of eosin Y (2′,4′,5′,7′-tetrabromofluorescein, 99% pure; Sigma Chemical Co., St. Louis, MO); α-(4-Pyridyl-1-oxide)-*N*-*tert*-butyl nitrone (4-POBN) (99% pure; Aldrich Chemical Co., Milwaukee, WI); MEA (99% pure; Fluka Chemie AG, Buchs, Switzerland); 1,4-diazabicyclo[2.2.2.]octane (DABCO) (Sigma Chemical Co.); histidine (J. T. Baker Chemicals BV, Deventer, The Netherlands); sodium azide (Merck, Darmstadt, Germany). Other chemicals used in making buffer solution came from J. T. Baker Chemicals BV. All buffer solutions were made from powder 1 day before experiment. Water was filtered through a Millipore Milli-Q system (18 MΩ). The MEA solutions were made as quickly as possible and immediately before use to minimize oxidation by oxygen. Argon gas of the highest purity (oxygen content < 0.5 ppm; Hoekloos BV, Schiedam, The Netherlands) was used.

Radical quenchers in solution

Several commonly known singlet oxygen scavengers (5 mM histidine, 5 mM sodium azide, and 100 mM DABCO; Mason and Rao, 1990; von Trebra and Koch, 1982) and general radical quenchers (100 mM MEA-hydrochloride; Sheetz and Koppel, 1979; and 100 mM 4-POBN; Buettner and Mason, 1990) were tested for their effects on the photobleaching of fluorescein in solution. Flushing fluorescein solution for 15 min with argon gas was used to examine the effect of purging oxygen on photobleaching. Each quencher was dissolved in phosphate-buffered saline (PBS) with the pH adjusted to 8.0. A fluorescein solution was made by dissolving the free acid form of fluorescein in PBS. All of the quencher solutions in the study were used with a final fluorescein concentration of 10 nM. For each experiment, there was a "dark control" and "PBS control," and a quencher solution under study. "Dark control" referred to fluorescein in PBS not exposed to the bleaching source. "PBS control" referred to fluorescein in PBS exposed to the same excitation light source as the quencher solutions under study.

A Leitz (Leica, Wetzlar, Germany) DM epifluorescence microscope with a 100-W mercury arc lamp and a 450–490-nm excitation filter block was used as a bleaching light source. The objective was removed so that a

column of light impinged upon the quartz cuvette containing the solution. A sample was exposed to continuous excitation light, and the fluorescence intensity was measured before the first exposure and again at 10-min intervals on a spectrofluorometer (SPF-500; SLM Instruments, Urbana, IL) for a total duration of 90 min. The cuvettes were mixed before each measurement. The spectrofluorometer was equipped with a xenon lamp, and the emission and excitation monochromator wavelengths were set to 490 ± 4 nm and to 512 ± 4 nm, respectively. All of the instrument settings were kept constant throughout the experiment.

For each sample, the intensity readings were plotted against time. The low fluorescein concentration allowed the bleaching curve to be described by a single-exponential function $f(t) = Ae^{-\kappa t}$, where κ is the rate of photobleaching, and A is fluorescence intensity at $t = 0$. The κ values of different samples were normalized to those of the dark and PBS control samples such that the κ values ranged between 0 (no photobleaching) and 10 (photobleaching of unprotected fluorescein in PBS).

Mercaptoethylamine in solution and in microscopy

Mercaptoethylamine was one of the scavengers tested in solution as described above. To assess its effect on closely packed and bound fluorescein in microscopy, the same concentration of 100 mM MEA in PBS was used as an embedding medium for microscope preparation. The solution of MEA in PBS was made fresh immediately before use.

Ficoll-isolated human lymphocytes on glass slides were stained by direct fluorescence in in situ hybridization using fluorescein-labeled probes specific for the centromeric region of chromosome 1. After in situ hybridization the preparation was counterstained with diamidino phenylindole and embedded with or without 100 mM MEA in PBS under a coverslip.

The imaging system consisted of a Leitz Aristoplan fluorescence microscope equipped with a 100-W mercury arc lamp and a charge-coupled device camera (series CH250; Photometrics, Tucson, AZ) with a Kodak (Rochester, NY) KAF-1400 chip of 1348 × 1035 pixels and a 12-bit dynamic range. A filter block with an excitation bandwidth of 450–490 nm, a dichroic mirror of 510 nm, and a long-pass emission filter of 520 nm were used for fluorescein-stained specimens. The microscope was adjusted to Köhler illumination and checked for flat-field illumination with uranyl glass. The shutter in the charge-coupled device camera and a mechanical shutter in the excitation light path were computer-controlled for the desired on-chip integration and duration of illumination, respectively. A Macintosh IIfx computer served as a host computer, directly controlling the shutters and image acquisition.

An object of interest was located by using low intensity and brief duration of UV (340–380 nm) excitation to detect the diamidino phenylindole counter-stain of a nucleus. Because fluorescein absorbs very poorly in this UV region, photobleaching of fluorescein was minimal. For each object of interest, a series of images was acquired under continuous steady illumination over a period of 240 s and with a blue (450–490 nm) excitation filter set. Each image was integrated for 0.3 s at 1-s intervals for the first 30 s and later at 12-s intervals. For determination of an accurate and unbiased total integrated intensity of each image, careful segmentation and background correction were done as follows. For background subtraction, the part of the gray value intensity image, $I(x,y,t)$, outside the area of the object was used. The background mask was found by first applying a gradient filter to $I(x,y,t = 0)$ and then thresholding the resultant image to locate the area with the magnitude of the gradient close to zero (i.e., the background area). This area was then used as an image mask for the subsequent images in the same series, and a mean value for each gray value intensity image under the background mask multiplied by the area of the whole image was subtracted from the total integrated intensity. The final scalar value of each image was the total integrated intensity after the background correction. A bleaching curve was made by plotting intensity values of all the images in a series against time.

Triplet population of eosin

The study of the triplet excited state population by means of time-delayed fluorescence spectroscopy may provide useful information concerning the effect of MEA on the photobleaching of fluorescein. However, this type of study could not be applied in the case of fluorescein because of its low triplet quantum yield. Therefore, eosin, which is the tetrabrominated form of fluorescein, was chosen for its high intersystem crossing quantum yield ($\Phi_{S^* \rightarrow T^*} = 0.64$ in aqueous solution at pH 7.2; Nemoto et al., 1969) and its similarity in photo-induced reactions (Koizumi and Usui, 1972).

Eosin solutions were made by dissolving the free acid form of eosin Y in 0.01 M phosphate buffer (PB: pH 7.6). The final concentration of eosin was 5 μM. The sample solution was placed in a quartz cuvette closed by a rubber stopper and was flushed with argon through inlet and outlet needles. For those samples to which MEA/PB solution was added, the eosin solution was first flushed with argon for 20 min, and MEA/PB solution was injected into the cuvette with a calibrated Hamilton needle (Hamilton Bonaduz AG, Bonaduz, Switzerland). The sample was then continuously flushed with argon for another 10 min. The triplet population was measured as a function of MEA concentration and the degree of degassing.

To measure the triplet population of eosin in the presence and absence of MEA, a luminescence spectrometer (LS 50S; Perkin-Elmer, Beaconsfield, England) was employed in its time-delayed mode. The spectrometer was equipped with a xenon discharge lamp, which could generate 20 kW power in a 8-μs pulse (full width half-maximum, FWHM), and with a red-sensitive (200–900 nm) photomultiplier (model R928; Hamamatsu). The spectrum of time-delayed luminescence emission of eosin in the study by Garland and Moore (1979) was used as a reference. The sample was excited at 500 nm with a slit width of 15 nm. The time-delayed luminescence signal was detected after a 100-μs delay and integrated for 600 μs between 530 and 800 nm with an emission slit of 15 nm. The prompt fluorescence was also measured for each sample with excitation at 515 nm, and emission from 530 to 800 nm, with both slit widths set to 2.5 nm. The signals were not corrected for the photomultiplier's spectral sensitivity. Absorption spectra were measured with a spectrophotometer (Ultrospec II; Pharmacia LKB, Uppsala, Sweden).

Laser flash photolysis

Two different laser flash photolysis setups were used to study the effect of MEA on the triplet excited state of fluorescein, and each had different advantages associated with this study. In the first flash photolysis setup (setup 1), with each laser flash, the T-T absorption change at a selected wavelength was recorded in real time with a fast digital oscilloscope. Thus a complete triplet decay curve as a function of time was recorded after each laser shot. In the second setup (setup 2), a spectrum as a function of wavelength at a specified time after a laser flash was acquired. Therefore, setup 1 was used to study the kinetics of the triplet population, and setup 2 was used to study the spectral changes caused by photoexcitation.

In setup 1, a high-pressure 500-W xenon lamp generated a probing pulse with a duration of 200 μs and a power of 10 kW/pulse. Shortly after the onset of the xenon lamp, a pulse of 15 ns (FWHM) at 248 nm was delivered by a KrF excimer laser. The laser and probing light source were placed at a right angle. Monochromators were placed in front of the xenon lamp and the photomultiplier. Each monochromator was set to transmit at 560 nm so as to determine the fluorescein triplet absorption change (Lindqvist, 1960). A sample was placed in a quartz cuvette of 1 × 1 cm, and the transient signal was recorded by a digital oscilloscope interfaced to a computer. Each sample was exposed to five laser flashes, and the transient absorption change, Δabsorbance ($\log(I_0/I)$), was derived from the transmittance of the solution to the 560 nm probing light before (I_0) and after (I) the laser flashes. The steady-state absorption spectrum was measured before and after the laser flashes to detect whether any permanent chemical changes had been induced by the laser flashes.

In setup 2 (see also Roest et al., 1994), a pulse of 7 ns (FWHM) with an energy of about 10 mJ/shot at 308 nm was delivered by a Lambda-Physik (Göttingen, Germany) EMG 101 Xe/HCl excimer laser. A 450-W

high-pressure pulsed xenon lamp was used as a probing source and was arranged in a right-angle geometry to the laser. The transmittance of the solution was detected at a right angle to the laser and collected by an optical fiber, which led to a spectrograph in which the light was dispersed by a grating (150 grooves/mm) onto a micro-channel plate-intensified diode array detector. A spectral range from 270 to 870 nm was covered with a bandwidth of 5 nm. The detector was gated with a width of 100 ns. Spectra were averaged over 20 laser flashes to improve the signal-to-noise ratio. Sets of spectrum were acquired at 1 μs after the laser flash and subsequently at 3-μs time intervals.

The sample preparation was identical for both setups and samples were freshly made 1 day before experiments. A 50 μM fluorescein solution was made by dissolving the free acid form of fluorescein in a 0.01 M phosphate buffer (pH 7.75). Immediately before each measurement, a MEA solution was made by dissolving MEA-hydrochloride in 0.01 M phosphate buffer (final pH 7.8). Because MEA absorbs between 240 and 250 nm, the concentration of MEA (50 μM) was chosen such that it was low enough to minimize its filtering effect and high enough to observe its quenching effect. All samples were flushed with argon continuously for 40 min. In the fluorescein sample to which MEA was added, 1.9 ml fluorescein solution was first flushed with argon for 20 min, then 0.1 ml of MEA solution was injected into the cuvette with a calibrated Hamilton needle, and the sample was then flushed with argon for another 20 min. In the sample where MEA was not used, 0.1 ml of PB was injected into the cuvette to keep the concentration of fluorescein solution constant throughout the experiment. The final concentrations of MEA and fluorescein were 250 μM and 47.5 μM, respectively.

TABLE 1 Effects of various radical quenchers on photobleaching of 0.01 μM fluorescein solution

Method	Conc. (mM)	κ*
O_2 removal		
Ar continuous flush	—	4.5
Ar 15-min flush	—	5.4
Singlet O_2 scavengers		
Histidine	5	9.2
NaN_3	5	11.5
DABCO	100	0.0‡
General radical quenchers		
MEA	100	0.0
4-POBN	100	0.0§
Combinations		
Ar + cysteamine	100	0.0
Controls		
PBS	—	10.0
Dark control	—	0.0

*For $\kappa = 0$, no photobleaching; $\kappa = 10$, photobleaching of unprotected fluorescein in PBS; $0 < \kappa < 10$, reduced photobleaching rate; $\kappa > 10$, increased photobleaching rate.
‡A reduction in fluorescence quantum yield by 50% was observed.
§A reduction in fluorescence quantum yield by 85% was observed.

RESULTS

Radical quenchers in solution

Flushing the solution with argon gas reduced photobleaching (Fig. 3 *a*), demonstrating the involvement of oxygen in photobleaching. With the exceptions of DABCO, 4-POBN, and MEA, none of the singlet oxygen scavengers or free radical quenchers used had any detectable effect on photobleaching at the concentration tested (Table 1). It must be noted that none of the tested scavengers or quenchers are necessarily specific for a particular radical, but may react with different radicals at different rates. Because singlet oxygen radical is usually the primary or secondary intermediate from photochemical reactions between excited singlet or triplet dye and oxygen (D-O reactions), the lack of the effect of these scavengers and quenchers on photobleaching suggests that singlet oxygen radical production may not be the major cause of photobleaching of fluorescein. This observation is in agreement with that of Johnson et al. (1982). In their study, heavy water (D_2O), which is known to prolong the lifetime of singlet oxygen from 4.2 μs in water (H_2O) to 55 μs in D_2O, did not have any effect on the photobleaching behavior. The results of DABCO and 4-POBN seem to contradict the foregoing observation, because they are known as singlet and peroxyl radical quenchers, respectively. Because they induced a considerable reduction in fluorescence quantum yield at the concentration tested (see Table 1), they were not investigated further.

MEA in solution and in microscopy

MEA was the only compound effective as an inhibitor of photobleaching without quenching the steady-state fluorescence (Table 1). In the presence of 100 mM MEA, the fluorescence intensity of the fluorescein solution (Fig. 3 *b*) was constant throughout a 90-min exposure to the bleaching

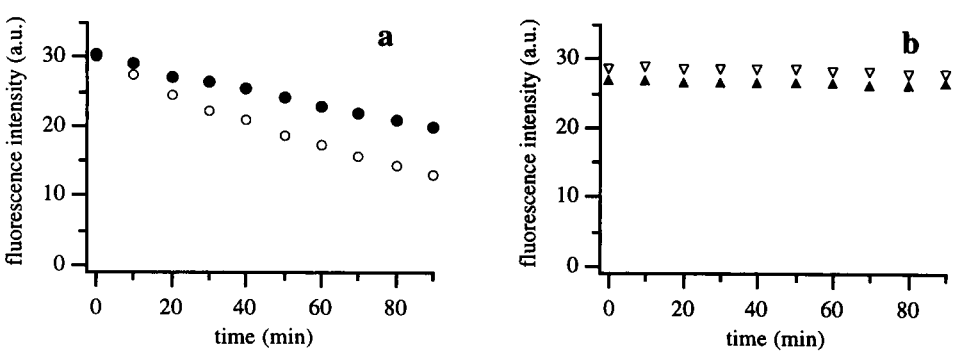


FIGURE 3 Photobleaching curves of 0.01 μM fluorescein in PBS (pH 7.6). (*a*) exposed to the bleaching light source, with (●) and without (○) 15 min argon (PBS control). (*b*) With (▽) 100 mM MEA and exposed to the bleaching light source and without (▲) 100 mM MEA and not exposed to the bleaching light source (dark control).

light source. At the same time, the fluorescence quantum yield (i.e., the fluorescence intensity at the first time point) was identical to that of the PBS control. According to the absorption spectrum (data not shown), a solution of 100 mM MEA showed no absorbance at wavelengths of >300 nm. When the same solution was used to embed fluorescein-labeled specimens for microscope measurement, the bleaching process was slowed down considerably compared to the PBS-embedded control sample (Fig. 4).

Quenching of the triplet excited-state population of eosin by MEA

The time-delayed luminescence spectrum of eosin had two broad bands. The band centered at 540 nm in Fig. 5 *a* arose from delayed fluorescence, whereas the band at 680 nm was due to phosphorescence. As shown in Fig. 5 *a*, the triplet state population of eosin was completely quenched by oxygen in the air-saturated eosin solution ($[O_2] \approx 250$ μM). The triplet population was increased as more oxygen was displaced by argon. When MEA was added to the argon-deoxygenated eosin solutions, the triplet state population (i.e., both the thermally activated delayed fluorescence and phosphorescence) decreased with increasing concentrations of MEA. The oxygen and MEA concentrations affected neither the prompt fluorescence (Fig. 5 *b*) nor absorption spectrum of eosin (data not shown).

Quenching of the triplet excited-state population of fluorescein by MEA

The triplet decay kinetics of fluorescein samples with and without MEA was studied using setup 1. The absorption spectrum of the triplet excited-state fluorescein in an aqueous medium at room temperature at wavelengths of >540 nm was established by Lindqvist (1960). Fig. 6 shows the decay of the triplet absorption at 560 nm for samples with and without MEA. These curves are clearly different and show a faster decay in the case of the sample with MEA. Note that the negative signal during the first 5 μs (Fig. 6) arose from the saturation of the photomultiplier by the strong fluorescence. The shape of the decay curves was not only determined by the first-order decay, but also by the second-order triplet-triplet reactions caused by the high concentration (50 μM) of fluorescein solution. Because of the low quantum yield of intersystem crossing of fluorescein, a high-concentration solution could not be avoided. Because the initial triplet concentration was unknown, the second-order process could not be accounted for properly in the numerical analysis of the decay curves. Therefore, the second-order process was neglected and a single exponential function was fitted to the curve as the first-order approximation.

Based on the decay rate constants obtained in this manner, a quenching rate was estimated using the relationship

$$k_{obs} = k_{T^*} + k_q \times [Q].$$

The observed triplet decay rate in the presence of MEA (k_{obs}) was 4.7×10^4 s^{-1}, and that in the absence of MEA (k_T*) was 3.04×10^4 s^{-1}, and [Q] was the concentration of the quencher molecules, i.e., [MEA] = 250 μM. The quenching rate, k_q, was then calculated to be about 6.6×10^7 M^{-1} s^{-1}.

The absorption and fluorescence spectra (at 490 nm of excitation) did not reveal any sign of interaction between MEA and the singlet ground and excited states of fluorescein at 250 μM MEA (data not shown).

Quenching of the radical forms of fluorescein by MEA

The transient existence of dye radicals was studied using setup 2. Lindqvist (1960) identified and studied the appearance and disappearance of the semireduced and semioxidized forms of fluorescein. In that study, performed under acidic and mildly alkaline condition, the semireduced molecules absorbed predominantly at 355 nm and the semioxidized radicals at 428 nm. Both of these species can be clearly identified in Fig. 7, along with the triplet population at wavelengths of >540 nm. In the case of fluorescein solution alone (Fig. 7 *a*), the semireduced (R), semioxidized (X), and the triplet (T*) forms did not show any tendency of dramatic change over the 55-μs period. In contrast, in the presence of 250 μM MEA (Fig. 7 *b*), the semioxidized (X) population, whose transient absorption change centered about 428 nm, showed a rapid decrease over the 55-μs period. The triplet and semireduced populations also showed a slow decrease over time, as compared to the fluorescein solution without MEA. No attempt was made to extract rate constants from this figure, because the signal-to-noise ratio and the resolution along the time axis were poor.

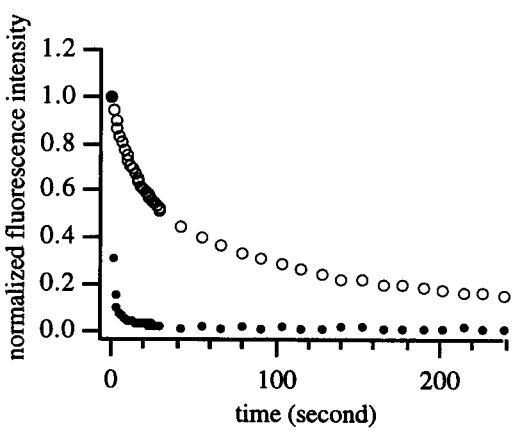


FIGURE 4 Photobleaching curves of fluorescein labeled directly to the centromeric region of chromosome 1 of human lymphocytes. ○, Photobleached sample when embedded in 100 mM MEA/PBS. ●, Sample embedded in PBS only.

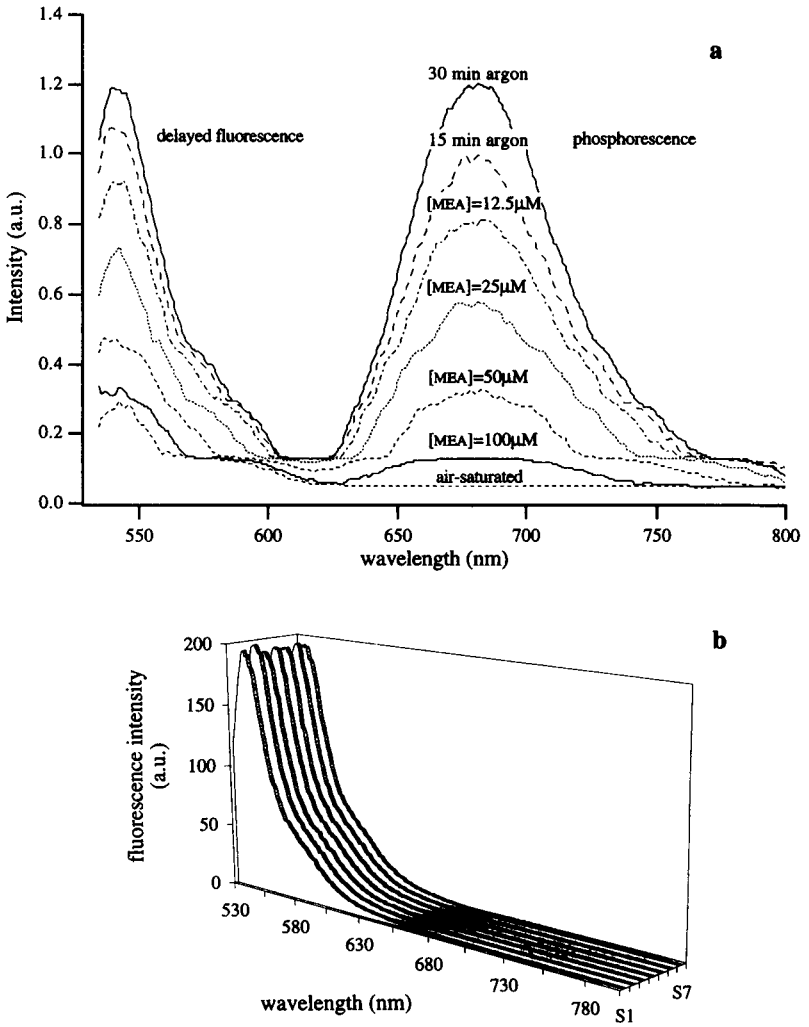


FIGURE 5 Luminescence emission spectra of eosin in aqueous solution. (*a*) The triplet excited state population of eosin as a function of oxygen and MEA concentration. See the main text for the detailed treatment of each sample. (*b*) The prompt fluorescence emission spectrum for each sample in *a*. S1–S7 designate the samples in the order listed from the top to the bottom curve in *a*.

The semioxidized and semireduced molecules were formed immediately through the D-D reactions at the onset of the laser flash. This was due to the relatively high concentration of fluorescein, which accelerated the D-D reactions (Usui et al., 1965; Song et al., 1995). The large differences in the transient absorption of these three species were primarily due to the large differences in their extinction coefficients, as measured by Lindqvist (1960), to be 5 $\times$ 10^4 for the semioxidized form (at 428 nm), 3 $\times$ 10^4 for the semireduced form (at 355 nm), and 1 $\times$ 10^4 M^{-1} cm^{-1} for the triplet excited-state molecules (at wavelengths > 540 nm).

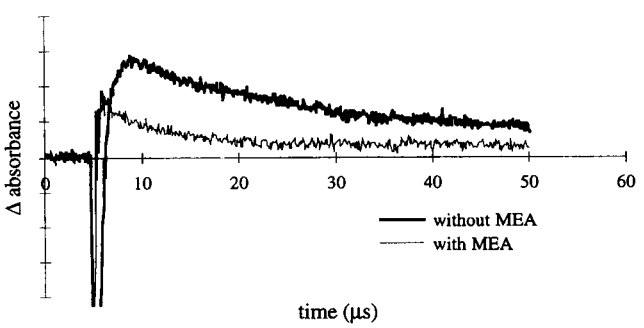


FIGURE 6 Transient decay kinetics of triplet excited state of fluorescein with (*thick trace*) and without (*thin trace*) of MEA. [Fluorescein] = 47.5 μM, [MEA] = 250 μM, probing wavelength = 560 nm, laser excitation = 248 nm.

In the spectral region between 440 and 530 nm, there was possibly a combination of the ground-state absorption, transient triplet absorption, and thermally activated delayed fluorescence, which resulted in a mixture of apparent positive and negative changes in absorbance. This region was not analyzed further.

DISCUSSION

Photobleaching protection by MEA

The results have demonstrated that MEA protects fluorescein against photobleaching. The difference in the degree of protection of free (Fig. 3) and bound (Fig. 4) fluorescein may arise from the difference in fluorescein concentration and accessibility. In solution, the concentration of fluores-

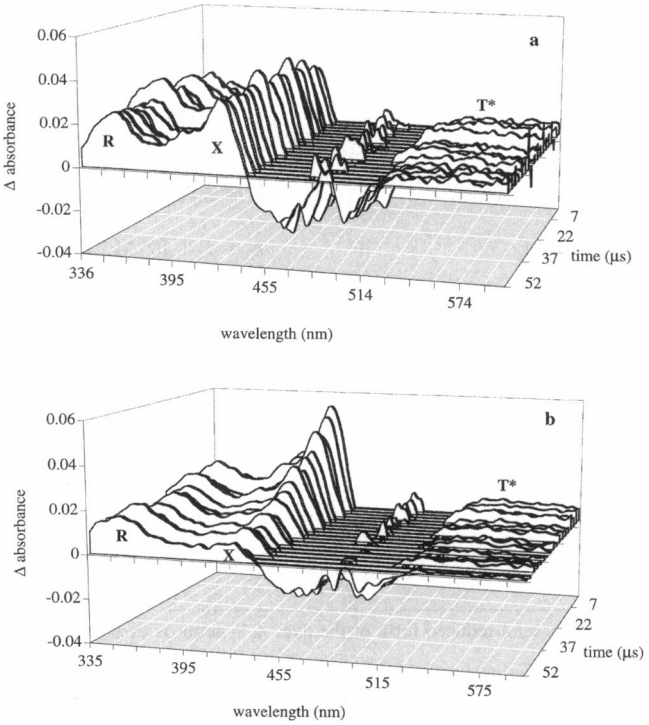


FIGURE 7 The transient existence of the triplet excited state (T*), semireduced (R), and semioxidized (X) radical forms of fluorescein. (*a*) The transient absorption change without MEA, and (*b*) with 250 μM MEA. [Fluorescein] = 50 μM, laser excitation = 308 nm.

cein was low (0.01 μM), the MEA concentration was high, and both fluorescein and quencher molecules were freely diffusible. Under these conditions, the triplet-triplet and triplet–ground-state reactions were minimized. The protective reaction between MEA and triplet-state fluorescein must compete with the reaction between fluorescein and oxygen. At [MEA] ≫ [O_2], this competition favors the reactions between MEA and fluorescein. In microscopy, fluorescein molecules are immobilized and cluster on small cellular targets. The decreased intermolecular distance between dye molecules increases the probability of D-D reactions (Song et al., 1995). This concentration quenching phenomenon has also been investigated by Robeson and Tilton (1995), using a very different approach, and they came to a similar conclusion. The protective reaction between MEA and fluorescein must compete with both the D-D and D-O reactions (Usui et al., 1965; Lindqvist, 1960; Song et al., 1995). Furthermore, fluorescein molecules in solution are more accessible to MEA from all directions, whereas fluorescein molecules chemically bound to the cellular targets are less accessible in microscopy. For these reasons the reduction in photobleaching caused by MEA was less complete in microscopy than in solution.

Pathway of MEA protection

Koizumi and Usui (1972) have shown that eosin and fluorescein have very similar photosensitized reactions, and only rates are different. Eosin was therefore chosen to study the effect of MEA on the triplet excited-state population. As shown in Fig. 5 *a*, the triplet-state population of eosin was completely quenched by oxygen in the air-saturated eosin solution ([O_2] ≈ 250 μM), demonstrating the efficiency of D-O reactions. When the oxygen concentration was decreased by flushing with argon, D-O processes were reduced and the triplet-state population was built up. When MEA was added to the well-degassed eosin solution, the triplet-state population was not further increased. On the contrary, with successive increases in the concentration of MEA, the triplet population decreased. The effect of MEA on the triplet state eosin was, therefore, not through the complete removal of residual oxygen by MEA, but rather through a direct interaction with the triplet-state molecules. The dye-quencher (D-Q) reaction became dominant. This result demonstrated that both D-O and D-Q processes affected the triplet population of eosin.

The singlet excited-state population was not affected by the change in the concentrations of oxygen and MEA, because the fluorescence intensity (Fig. 5 *b*) of each sample remained constant despite the change in the concentrations of oxygen and MEA.

The result of MEA on eosin provided sufficient evidence to justify more complicated flash photolysis experiments in which a direct proof of MEA reacting with triplet-state fluorescein could be obtained.

MEA quenching of the triplet and radical forms of fluorescein

The kinetics of the triplet excited state of fluorescein were shown to be strongly affected by the presence of MEA (Fig. 6). As already mentioned, the necessarily high concentration of fluorescein and photon saturation of prompt fluorescence on the photomultiplier precluded extraction of triplet lifetimes from the kinetics curves. The triplet lifetime extracted by using the first-order approximation of the second-order process clearly indicated the difference in the triplet kinetics in the absence and presence of MEA. It demonstrated that MEA altered the triplet excited-state kinetic behavior. It should be emphasized that it is the difference in the triplet lifetimes (with and without MEA) that is of major importance in this study. The difference in the triplet lifetimes with and without MEA is better expressed through the estimated quenching rate. Although several concentrations of MEA were usually necessary to derive a more accurate k_q from a Stern-Volmer plot, it is sufficient for the purpose of this study to demonstrate the differences in the triplet lifetimes as expressed in the large quenching rate.

The formation of semireduced and semioxidized radicals is the consequence of triplet-triplet and triplet–ground-state reactions (Table 2). The semioxidized radicals, being a stronger oxidizing agent than the triplet state (Grossweiner and Kepka, 1972; Lindqvist, 1960), reacted with the reductant, MEA, very rapidly (Fig. 7 *b*). This phenomenon,

TABLE 2 Rate constants of singlet and triplet excited state reactions of fluorescein[a]

No	Reaction	Description	Rate Constants
	$S + h\upsilon \rightarrow S^*$	Absorption	($\sigma_a = 3.06 \times 10^{-16}$ cm^2)[b]
	$S^* \rightarrow S + h\upsilon'$	Fluorescence emission	$k_d = 2.134 \times 10^8$ s^{-1}[c]
	$S^* \rightarrow S$	Internal conversion	
	$S^* \rightarrow T^*$	Intersystem crossing	$k_{isc} = 6.6 \times 10^6$ s^{-1}
1	$T^* \rightarrow S$	Radiationless deactivation	$k_1 = 50$ sec^{-1}[d]
2	$T^* + T^* \rightarrow T^* + S$	Triplet quenching	$k_2 = 5 \times 10^8$ M^{-1}s^{-1}
3	$T^* + S \rightarrow S + S$		$k_3 = 5 \times 10^7$ M^{-1}s^{-1}
4	$T^* + T^* \rightarrow R + X$	Electron transfer	$k_4 = 6 \times 10^8$ M^{-1}s^{-1}
5	$T^* + S \rightarrow R + X$	Electron transfer	$k_5 = 5 = 10^7$ M^{-1}s^{-1}
6	$T^* + X \rightarrow S + X$	T* quenching by X	$k_6 + k_7 = 1 \times 10^9$ M^{-1}s^{-1}
7	$T^* + R \rightarrow S + R$	T* quenching by R	k_7
8	$T^* + O_2 \rightarrow S + O_2$	Physical quenching by O_2	$k_8 = 1.56 \times 10^9$ M^{-1}s^{-1}[e]
9	$T^* + O_2 \rightarrow X + HO_2$ (or O_2^-)	Chemical quenching by O_2	$k_9 = 1.4 \times 10^8$ M^{-1}s^{-1}
10	$T^* + Q \rightarrow S + Q$	See text	
11	$T^* + Q \rightarrow R + Q_{ox}$		
12	$R + Q_{ox} \rightarrow S + Q$		
13	$X + Q \rightarrow S + Q_{ox}$		

S = ground state dye; S* = singlet excited state dye; T* = triplet excited state dye; R = semi-reduced form of the dye; X = semi-oxidized form of the dye; O_2 = oxygen.

[a]Part of this table was published in Song et al. (1995). For completeness, it is included in this report.

[b]σ_a (3.06×10^{-16} cm^2/molecule for fluorescein at 488 nm), k_d and k_{isc} were quoted from Tsien and Waggoner (1989).

[c]k_d is the combined rate of radiative (k_f) and nonradiative (k_{ic}) S*−S transitions, respectively.

[d]The rate constants k_1 to k_9 were quoted from Lindqvist (1960) and Kasche and Lindqvist (1964).

[e]In the original work of Lindqvist and Kasche (Lindqvist, 1960; Kasche and Lindqvist, 1964), $^3O_2$ and $^1O_2$ were not separately investigated. Here they are quoted without modification.

combined with the fact that fluorescence quantum yield was not affected by the presence of MEA, can be very well described by the reaction schemes proposed in earlier studies of other reducing agents (Grossweiner and Kepka, 1972; Lindqvist, 1960; Koizumi and Usui, 1972), namely, reactions 10 to 13 in Table 2, where Q is a quencher molecule (i.e., reductant such as MEA) and Q_{ox} is the oxidized form of Q. T* is postulated to be quenched in a fast multiple-step process (reaction 10), or by a fast two-step process (reactions 11 and 12). The net result is the same: there is no loss of S in this D-Q scheme. The X produced by D-D reactions further reacts with Q (MEA) and thus reverts to the ground state (reaction 13). If the D-Q processes can compete favorably with the D-O and D-D processes, then T* molecules could be restored to the ground state by the D-Q mechanism.

Competition among D-O, D-D and D-Q mechanisms

In fluorescence microscopy, dye molecules are often densely bound to a cellular target. The small intermolecular distance between dye molecules can be expressed in a high local concentration of dye. The possible fate of T* molecules and the radical forms of fluorescein in the absence or presence of MEA (or other reducing agents acting as triplet quenchers where $[Q] \gg [O_2]$) may be proposed in accordance with the conclusions of Usui et al. (1965) and summarized as in Table 3.

In the practical microscopy situation, where oxygen is generally present, photobleaching can be characterized by the competition between D-O and D-D reactions (case 3 in Table 3). If a highly efficient triplet quencher ($k_q \geq 10^9$ M^{-1} s^{-1}) can be found and applied, the D-Q reactions can compete favorably with the D-D and D-O reactions so as to achieve protection from photobleaching (case 7 in Table 3). It should be mentioned that the competition between D-Q and D-O reactions is not simply determined by the relative concentrations of the quencher and oxygen. More importantly, the quenching efficiency is determined by the energy

TABLE 3 A summary of possible experimental conditions and the corresponding photobleaching mechanisms of fluorescein

Case	Experimental conditions →	Mechanism(s)
1	Oxygen present and [dye] low*	D-O dominates
2	Oxygen absent and [dye] high	D-D dominates
3	Oxygen present and [dye] high	D-O competes with D-D
4	Oxygen absent, Q present, [dye] low	D-Q dominates
5	Oxygen absent, Q present, [dye] high	D-Q competes with D-D
6	Oxygen present, Q present, [dye] low	D-O competes with D-Q
7	Oxygen present, Q present, [dye] high	D-O, D-D, and D-Q compete

*For fluorescein, this threshold is 5 μM (Koizumi and Usui, 1972; Lindqvist, 1960) under their experimental conditions.

level of the quencher relative to the lowest triplet energy level of the dye.

MEA as an antifading agent

From the viewpoint of a microscopist and cell scientist, MEA possesses some important qualities as a potential photobleaching protector. Unlike the reducing agents (*p*-phenylene-diamine, EDTA, or allyl-thiourea) studied by Lindqvist (1960) and Koizumi et al. (Koizumi and Usui, 1972) which, to different degrees, reduce the quantum yield of fluorescein at high concentrations of the quenchers and/or react with the ground state at low $[O_2]$, MEA does not reduce the quantum yield of fluorescence, as was demonstrated by Figs. 3 *b* and 5 *b* for both fluorescein and eosin. Furthermore, its transparency in the visible region of the spectrum implies a low background if added to the embedding medium.

MEA, as a photobleaching protector, performs a double function. Its readiness to be oxidized to cystamine by the oxygen from the environment made it a very unstable molecule and at the same time a good oxygen "consumer." Once MEA is oxidized to cystamine (on standing overnight), it loses its protecting ability (data not shown). This implies that SH is the active group in the photobleaching protection process.

CONCLUSIONS

The essence of this study was to provide the photophysical evidence for the conclusion of our previous study (Song et al., 1995), that among many possible photosensitized reactions the triplet excited state of fluorescein plays a very significant role in the photobleaching of fluorescein in microscopy. Although mercaptoethylamine has many desirable qualities as a protecting agent in photobleaching of fluorescein, it serves in a more important way in this study as a window into the complex problems related to the photobleaching mechanism of fluorescein in microscopy. It was demonstrated that the photobleaching mechanism of fluorescein is explained at least in part by the accumulation of the long-lived triplet excited state of fluorescein and that by quenching the triplet excited state and semioxidized radicals without causing a net change in the ground state-population, photobleaching can be reduced. Understanding the photobleaching mechanisms through the action of MEA can enable us to better control the influence of the triplet excited state of fluorescein on photobleaching, and to design more photostable fluorophores and more efficient photobleaching protectors.

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