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Photoreduction of Resazurin in the Presence of Aliphatic Amines

Miguel G. Neumann,[a]* Carla C. Schmitt,[a] Carlos M. Previtali[b] & Sonia G. Bertolotti[b]

[a]Instituto de Química de São Carlos, Universidade de São Paulo, Caixa Postal 780,
13560-970 São Carlos SP, Brazil
[b]Departamento de Química y Física, Universidad Nacional de Río Cuarto,
5800 Río Cuarto, Argentina

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ABSTRACT

Photophysical and photochemical properties of the dye resazurin were measured. The quantum yields of fluorescence and triplet formation were determined to be 0.10 ± 0.01 and 0.2, respectively. The lifetimes of the singlet and triplet states are 1.2 ns and 30 μs. In the presence of aliphatic amines, resazurin is readily photoreduced to resorufin. This reaction proceeds through the triplet state and is inhibited by the presence of oxygen. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Dyes are used quite frequently as probes to analyze the behaviour of various types of systems,[1,2] especially those having microheterogeneous regions. Additionally, various biochemical and industrial properties can be determined using dyes by utilising the changes in color of the compounds upon oxidation or reduction. As the substrates often contain proteins or other amine-group-containing systems, it is very important to know the interactions between the probes and those amine groups. We have previously studied[3–7] the interaction of Methylene Blue and Safranine with compounds containing amino groups. Many dyes have a potential for use as polymerization photoinitiators,[8] especially those that have high absorption coefficients in the visible region, as is the case of resazurin (I).

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*To whom correspondence should be addressed.

Most of the dyes used as probes are cationic, which facilitates their binding to anionic sites; little work has been performed with anionic dyes derived from aromatic phenols. One dye of this type is resazurin, which has an oxidation potential that makes it an appropriate probe for the evaluation of various biochemical properties of milk,[9,10] sperm quality,[11,12] biochemical antioxidants,[13] etc. It has also been used in the analytical determination of ions such as Se(IV) and Pb(II)[14,15] and compounds derived from, or containing, aldehyde moieties.[16,17] In all these uses, resazurin is reduced to the closely related dye resorufin (**II**), changing its color from blue to red. Strangely enough, no photochemical study is known which evaluates the behaviour of this dye towards light. The potentiometric reduction of this dye with titanium (III) chloride is known and it was found that after an initial step in which the dye is reduced to resorufin, it is further reduced to dihydroresorufin.[18] The authors noticed that in the presence of light the reduction rate was larger, particularly for the methoxy substituted derivative.

In this communication we present some data on the excited states and photochemistry of resazurin in the presence and absence of oxygen and added amine.

EXPERIMENTAL

Resazurin and resorufin were obtained form Aldrich and used without further purification. All solutions were made in methanol (Merck, spectrophotometric or HPLC grade) and the measurements were performed at room temperature (25 ± 1°C).

Fluorescence spectra were measured on a Spex Fluoromax fluorimeter, and the singlet lifetime was determined by the single-photon timing technique using a CD-900 Edinburgh spectrometer operating with a low pressure nanosecond H_2 flash lamp at 25 kHz pulse frequency. Transient absorption spectra and transient decays were measured on an Applied Photophysics laser kinetic spectrometer. Excitation at 532 nm was performed using the second harmonic of a Nd-YAG laser (Spectron).

Photolyses were performed on an PTI photolyzer with a 150 W Xe lamp.

RESULTS AND DISCUSSION

Singlet and triplet states of resazurin

The UV-vis spectrum of resazurin in water is well known and shows a principal absorption at 598 nm and a lower intensity double peak at 380 nm.[19]

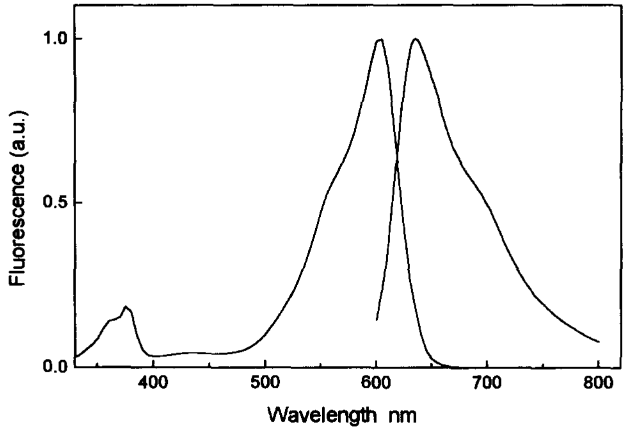


Fig. 1. Excitation ($\lambda_{em} = 636$ nm) and emission ($\lambda_{exc} = 570$ nm) spectra of resazurin in methanol. $OD_{602} = 0.13$.

These peaks can be assigned to the large ring $\pi\pi^*$ transition and to an $n\pi^*$ transition of the quinone group. In methanol the longer wavelength band is red-shifted (604 nm), whereas the lower wavelength band does not significantly change (377 nm). The fluorescence spectrum shows a maximum at 636 nm (Fig. 1), and is the same independent of the excitation being in the 604 or 377 nm region. The corrected excitation spectrum coincides with the absorption spectrum.

The fluorescence quantum yield for the dye in methanol has been determined to be 0.10 ± 0.01, using Cresyl Violet as reference.[20] The lifetime of the excited singlet state was measured and a value of 1.2 ± 0.1 ns was obtained in methanol.

Laser flash photolysis experiments show the presence of a transient species absorbing in the region of 300–500 nm. On the basis of oxygen and amine quenching experiments, this transient was ascribed to the triplet state. The spectra at various times after the laser pulse are shown in Fig. 2. The decay time, measured at 400 nm in deaerated solutions, is around 30 μs. In the presence of oxygen the quenching of this species is complete and no signal can be observed.

The quantum yield for the triplet should be relatively low, as the absorbance of the triplet in its main peak is around 0.025, when starting with dye concentrations around 10^{-5} M. Assuming that the absorption coefficients for the ground state and the triplet state at 375 nm are similar (as found for many dyes) and that all the dye in the light path is excited, the quantum yield can be estimated to be around 0.2.

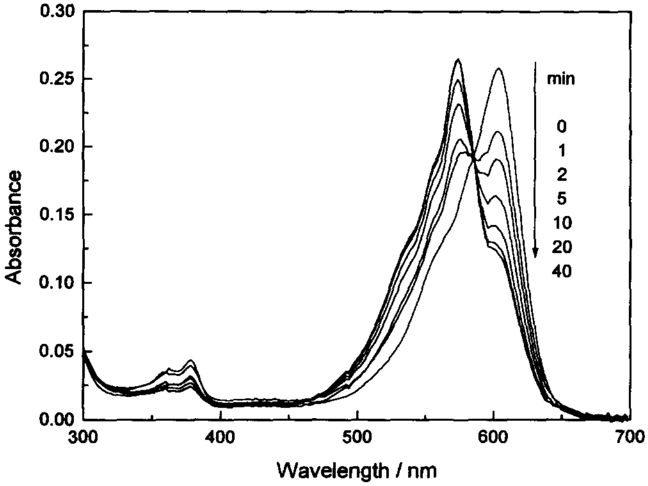


Fig. 2. Time evolution of the absorption spectrum of resazurin when irradiated at 600 nm in the presence of tri-*n*-propylamine 0.005 M in oxygen-free methanolic solution.

Photolysis of resazurin

As found for many other dyes,[21] resazurin can act as an efficient electron acceptor or donor in its excited state. Their excited state fluorescence is quenched efficiently by several electron donors, in particular aliphatic amines.

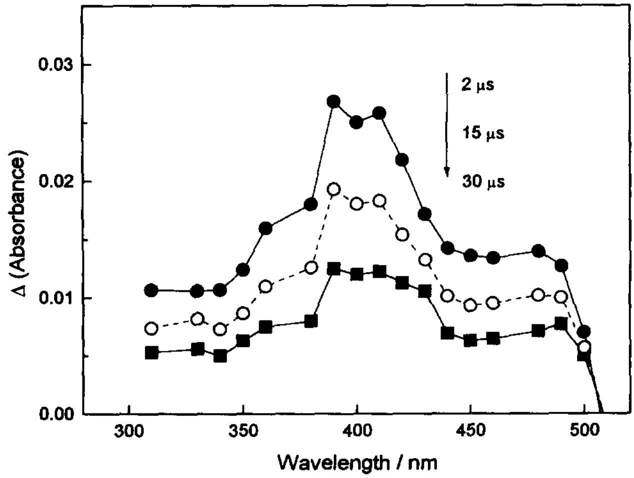
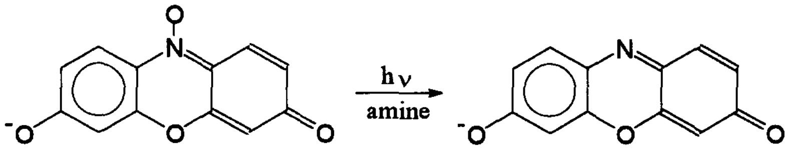


Fig. 3. Spectra of the triplet state of resazurin in methanol at different times after irradiation at 532 nm in oxygen-free solutions.

Resazurin is not affected by visible light in air equilibrated solutions, even in the presence of amines. However, in deaerated solutions when it is irradiated continuously at 600 nm, in the presence of tripropylamine the absorbance at 600 nm decreases slowly and a new absorption appears around 530 nm, as shown in Fig. 3. After exhaustive illumination, the spectrum of the irradiated solution is indistinguishable with that of resorufin, indicating the complete photoreduction of the original dye. The overall process may be written as



Resazurin **Resorufin**

I **II**

The final absorbance at 530 nm is around 20% higher than the initial absorbance of resazurin. Using the extinction coefficients at the maximum for both dyes (61,000 cm^{-1} M^{-1} for resazurin and 63,800 cm^{-1} M^{-1} for resorufin[19]) it can be estimated (within the confidence limits of the extinction coefficients) that for every consumed molecule of resazurin only one molecule of resorufin appears. This means that the photoreduction process only goes up to the cleavage of the N–O bond, and does not proceed further to the formation of dihydroresorufin, as found in electrochemical reductions.[18]

The very low concentration of TPA (0.005 M) suggests that the dye triplet may be involve in the reaction. We have investigated the triplet quenching by TPA, and the results in the form of eqn (1) are presented in Fig. 4.

$$\tau^{-1} = \tau_0^{-1} + k_q[\mathrm{TPA}] \qquad (1)$$

In eqn (1), τ_0 and τ are the triplet lifetime in the presence and absence of TPA, respectively, and k_q is the bimolecular triplet quenching rate constant. From the slope of the plot a value of 3.6×10^8 M^{-1} s^{-1} results for k_q. The fraction of triplets quenched at a given amine concentration, f, can be obtained from eqn (2).

$$f = \frac{k_q[\mathrm{TPA}]}{\tau_0^{-1} + k_q[\mathrm{TPA}]} \qquad (2)$$

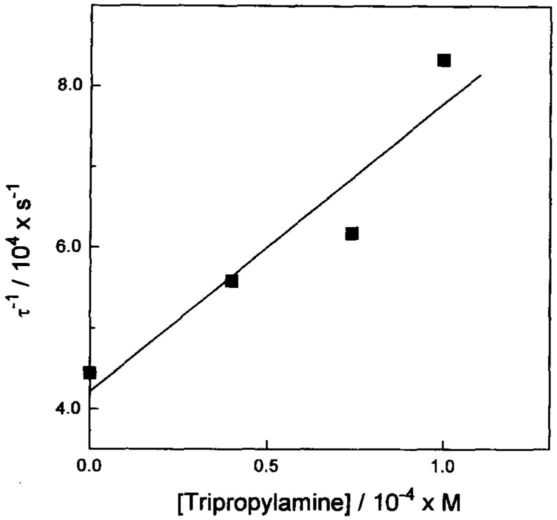


Fig. 4. Stern-Volmer plot for the quenching of resazurin by tri-*n*-propylamine in deareated methanolic solutions. Lifetimes were measured at 400 nm.

Accordingly, for the TPA concentration in the photolysis experiment, 0.005 M, and the kinetics parameters for the triplet quenching, a value of $f = 0.98$ is obtained. On the other hand, the corresponding quantity for the singlet state, even assuming a diffusional quenching rate constant, is practically zero. Therefore, it may be concluded that the photoreduction of resazurin by TPA most likely involves as a first step the interaction of the excited dye triplet with the amine.

Further work is in progress on the effect of the amine structure and the solvent on this reaction.

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