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The excited-states quenching of resazurin and resorufin by *p*-benzoquinones in polar solvents

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Abstract

The quenching of the excited singlet and triplet states of resazurin and resorufin by *p*-benzoquinones was investigated in methanol and in aqueous solution at pH 10. Fluorescence lifetimes were determined for the dyes and from the fluorescence quenching experiments rate constants were obtained for the singlet state quenching in MeOH. The rate constants were nearly diffusion controlled. Triplet state processes and transient absorption spectra were investigated by laser flash photolysis. Triplet quenching rate constants were obtained from the decay of the triplet at 825 nm for resazurin and at 700 nm for resorufin as a function of the quinone concentration. In the case of resazurin, the triplet quenching rate constants were lower than those of the singlet state. For resorufin, the values of the singlet and triplet were in the same order. From the transient absorption spectrum determinations it was concluded that the quenching proceeded by an electron-transfer reaction from the dye excited-states to the quinones. The results suggest that these dyes may function as very efficient photochemical-reductants.

Keywords: Resazurin; Resorufin; Benzoquinones; Excited-states; Photochemical-reductants; Electron-transfer

1. Introduction

Dyes are used quite frequently as probes to analyze the behavior of various types of systems [1], especially those having microheterogeneous regions. Additionally, various biochemical and industrial properties can be determined using dyes by utilizing the color changes of the compounds upon oxidation or reduction.

Resazurin is a phenoxazin-3-one dye widely used for testing various biological materials such as biochemical antioxidants [2]. Other example of its use is the "resazurin test"; in this test the reduction of the resazurin to resorufin (Scheme 1) depends on the ability of metabolically active spermatozoa. This reduction is manifested by a visible change in color from blue to pink, and this color change correlates significantly with concentration of motile spermatozoa [3]. Resorufin has also been used as a probe molecule to study the re-orientation of solvent molecules, and has shown an interesting chemistry and photochemistry in protic solvents which strongly depends on temperature, viscosity and structure of the solvent [4–6].

We have previously reported the photoreduction of dyes (flavines, safranine, resazurin and resorufin) by amines [7–11]. Many of the above mentioned dyes have a potential for use as polymerization photoinitiators due to high absorption coefficients in the visible region. It was found that a clean photodeoxygenation to resorufin occurs from the quenching of the triplet state of the dye [10]. This photodeoxygenation is dependent on the amine structure and is efficient only in the presence of tertiary aliphatic amines [11].

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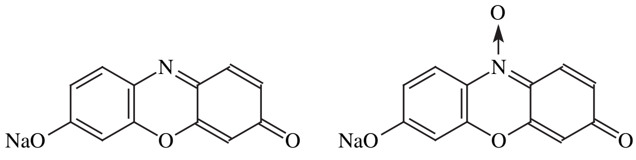

RESORUFIN RESAZURIN

Scheme 1.

The photoinduced deoxygenation of N-oxides has been reported previously for other molecules [12] and was found to proceed on irradiation of N-oxide alone or in the presence of suitable electron donors such as amines [13,14]. But in most cases the reaction is accompanied by side reactions and rearrangements of the N-oxide [15]. More recently, we found that the related dyes resazurin and resorufin, produce radicals in the presence of triethanolamine (TEOHA) that are efficient photoinitiators of aqueous acrylamide polymerization [16].

The one electron photooxidation of dyes has received less attention [17,18]. However, the quenching of excited-states of dyes by quinones is of interest due to the important role of the quinone moiety as an electron acceptor in photobiological process [19]. In this communication we present data on the interaction of the excited-states of the phenoxazin-3-one dyes resazurin and resorufin with *p*-benzoquinones as electron acceptors in alkaline water and methanol. Water was selected due to biological and environmental considerations while the organic solvent methanol was employed for comparative purposes. The quenching of singlet and triplet states was investigated and rate constants were determined. The nature of the quenching process was investigated by laser flash photolysis.

2. Experimental procedures

2.1. Chemicals

Resazurin and resorufin were obtained from Aldrich and used as supplied. The quinones, *p*-benzoquinone (BQ), methyl-*p*-benzoquinone (MQ), 2,5-dimethyl-*p*-benzoquinone (DMQ), 1-chloro-*p*-benzoquinone (ClQ) and duroquinone (tetramethyl-*p*-benzoquinone) (DQ) were obtained from various commercial sources. They were purified by recrystallization and/or sublimation when necessary. The solvent methanol (MeOH) was Sintorgan HPLC grade. Water was purified through a Millipore Milli-Q system.

2.2. Measurements

In water, both dyes are fluorescent only in their anionic forms at pH above 7.0. For their determination in aqueous solution the pH was adjusted by addition of NaOH (Aldrich, 99.9%) at 10 ± 0.1. In all cases the temperature was constant at 25 ± 1 °C.

Absorption spectra were determined on a Hewlett–Packard 6453E diode array spectrophotometer.

Steady-state fluorescence measurements were made using a Spex Spectrofluorimeter. Fluorescence quantum yields were determined relative to cresyl violet in methanol [20]. Fluorescence lifetime measurements were performed with an Edinburgh Instruments OB 900 time-correlated single-photon counting fluorometer. The singlet quenching rate constants were measured following the decrease of the lifetime by the quinone addition.

Transient absorption measurements were made using a laser flash photolysis equipment. The solutions were purged with argon for 30 min before their use. A Spectron SL400 Nd:YAG laser generating 532 nm laser pulses (~18 ns pulse width) was used for sample excitation. The laser beam was defocused in order to cover all the path length (10 mm) of the analyzing beam from a 150 W Xe lamp. The experiments were performed with rectangular cells with right angle geometry. The detection system comprises a PTI monochromator coupled to a Hamamatzu R666 PM tube. The signals were initially captured by an HP54504 digitizing oscilloscope where they were averaged and then transferred to a computer for storage and analysis. Measurements were performed in samples subjected to a continuous bubbling with high purity argon. For the measurement of the quenching rate constants a concentrate quinone solution was prepared in MeOH and a few microliters were added to solution in water.

Quantum yields were determined by actimonetic method using zinc tetraphenylporphyrin (ZnTPP) as reference. Values of $7.3 \times 10^4\ \mathrm{M}^{-1}\,\mathrm{cm}^{-1}$ and 0.83 were used for ε_T and ϕ_T of ZnTPP, respectively [21].

The molar absorption coefficients of triplet dyes were determined by the total depletion method [22] (Table 1). In both cases the negative absorption of the difference transient spectra matched the ground-state band. This is consistent with the lack of photoproduct formation under our conditions of laser experiments.

The voltammetric characterizations of the redox processes for all compounds were done with a potentiostat–galvanostat Autolab (Electrochemical Instruments), using a Pt disc as working electrode, a saturated calomel electrode as reference, and a large area Pt counter electrode, in a conventional three compartment Pyrex cell. Studies were carried out in ultrapure water (LABCONCO system) pH of 10 (NaOH; 0.1 M $NaClO_4$), and in methanol (0.1 M tetrabutylammonium hexafluorophosphate (TBAHFP, Aldrich) as supporting

Table 1
Photophysical parameters of resazurin and resorufin in MeOH and aqueous solution at pH 10

	Resazurin		Resorufin	
	MeOH	H_2O (pH 10)	MeOH	H_2O (pH 10)
Absorption λ_{max}/nm (ε/M^{-1} cm^{-1})	604 (45 000 ± 2200)	602 (56 000 ± 2800)	572 (50 000 ± 2500)	572 (55 000 ± 2700)
Fluorescence λ_{max}/nm	636	634	583	582
ϕ_F	0.11 ± 0.01	0.11 ± 0.01	0.53 ± 0.03	0.83 ± 0.01
τ/ns	1.1	0.7	4.5	2.8
ϕ_T	0.080 ± 0.010	0.070 ± 0.010	0.030 ± 0.005	0.04 ± 0.005
Triplet λ_{max}/nm (ε/M^{-1} cm^{-1})	825 (4600 ± 300)	825 (5400 ± 300)	700 (12 500 ± 500)	700 (15 000 ± 1000)

electrolyte). IR droop was corrected using a positive feedback technique.

The chlorinated quinones have a very slight solubility in water. Thus, the reduction potential in this media was estimated from a plot of E_0 red in water vs. E_0 red in MeCN, assuming that the change of solvation energy of quinones is small in the series. A plot of experimental reduction potentials and theoretical LUMO energies (AM1) of the studied quinone compounds showed a linear relationship [23] which agrees with our assumption.

3. Results and discussion

The resazurin UV–visible absorption spectrum in methanolic solution was similar to that reported [10] in aqueous solutions at pH 10. It consists of an intense absorption band at 604 nm ($\varepsilon = 45\,000$ M^{-1} cm^{-1}) and a weak band at 380 nm. The bands are assigned to the ππ* transition of the phenoxazin-3-one and to the weak nπ* transitions of the N-oxide.

The spectrum in water showed a dependence on the pH, but in the pH range 7.5–11 it is almost pH independent. A p*K* value of 5.5 has been reported from absorption at 602 nm vs. pH, which was assigned to the protonation of one of the oxygen atoms on the phenoxazin ring [11]. At 602 nm the absorption intensity increases linearly with the concentration (0.5–20 μM). This indicates that the aggregation of the dye is not appreciable in this concentration range.

The visible spectrum of resorufin was characterized by an intense band centered at 572 nm ($\varepsilon = 55\,000$ and 50 000 M^{-1} cm^{-1} in water and MeOH, respectively) with a shoulder at 535 nm. In water the absorbance and shape of the spectrum were unaffected in the pH interval 7.5–12, as in the case of resazurin. A p*K* value of 5.8 has been reported [11] from the absorbance at 572 nm against pH. This value is very close to that obtained for resazurin and was also attributed to the protonation of one of the oxygen atoms on the phenoxazin ring [11]. Similar spectral changes have been reported for resorufin by Flamigni et al [6]. In our study the absorption intensity increased linearly at 572 nm with the concentration (0.5–20 μM). This indicates that the aggregation of the dye is not appreciable in our working concentrations.

When excited at 602 nm, resazurin in water presented a fluorescence spectrum with an unstructured and broad band with a maximum at 634 nm. The fluorescence intensity, like the absorption, also presented a strong pH dependence, but the spectral shape remained unaltered. The emission intensity remained unchanged from pH 7.5 to 12, and a fluorescence quantum yield of 0.11 was determined in this pH range. In MeOH the maxima of the absorption and emission were 604 and 636 nm, respectively. The fluorescence quantum yield was 0.11.

Resorufin excited at 572 nm exhibited a strong fluorescence emission with a broad band centered at 583 nm and 582 nm in MeOH and water, respectively. This band remained almost unchanged between pH 7.5 and 12, with fluorescence quantum yield of 0.83 and 0.53 in water and MeOH, respectively.

The fluorescence lifetimes in water at pH 10 were 0.7 ± 0.1 ns and 2.8 ± 0.1 ns for resazurin and resorufin, respectively. In MeOH the lifetimes were 1.1 ± 0.1 ns and 4.5 ± 0.1 ns for resazurin and resorufin, respectively. The longer lifetime of resorufin, as compared with that of resazurin, is in agreement with the high fluorescence quantum yield. These values give radiative rate constants for the emitting state of 1.2×10^8 s^{-1} and 2.5×10^8 s^{-1} for resazurin and resorufin, respectively, which are consistent with an allowed ππ* electronic transition. The photophysical data for both dyes are summarized in Table 1.

3.1. Excited-states quenching by quinones

3.1.1. Singlet quenching

The fluorescence of resazurin and resorufin in MeOH was efficiently quenched by quinones. Bimolecular quenching rate constants (k_q^S) were determined from the Stern–Volmer (SV) plots of I_o/I or τ_o/τ vs. quinone concentration *[Q]*, where I_o and I stand for the fluorescence intensity, and τ_o and τ are the fluorescence lifetimes in the absence and the presence of quinone, respectively.

For low quencher concentrations, the Stern–Volmer plots determined from the fluorescence intensity showed a linear relationship. However, for the stronger quenchers, steady-state SV plots showed small positive deviations, indicating some contribution of static quenching. Nevertheless, in these cases SV plots based on lifetime measurements were linear, and quenching rate constants were evaluated from the slopes. The k_q^S values for the different quinones were collected and are shown in Table 2 for MeOH. It was not possible to measure the fluorescence quenching rate constants in water due to a very poor solubility of quinones (except *p*-benzoquinone and in this case the rate constant was nearly diffusion controlled).

For both dyes in MeOH, the rate constants approach a limiting diffusion value (1.2×10^{10} M^{-1} s^{-1}) for quinones of low reduction potential and decrease slightly when the reduction potential increases. This is in agreement with the expectation for an electron-transfer reaction from the excited dye to the quinones. The experimental redox potentials for quinones are summarized in Tables 2 and 3.

3.1.2. Triplet quenching

In the presence of quinones a shortening of the triplet lifetime of the dyes was observed (see Fig. 1). The bimolecular quenching rate constants (k_q^T) were determined from the experimentally measured first-order decay (k_{obs}) according to:

$$k_{obs} = k_o + k_q^T[Q]$$

where k_o is the decay rate constant in the absence of quinone. The values of k_q^T are included in Table 2 for MeOH and Table 3 for aqueous solutions at pH 10.

For resazurin in MeOH the k_q^T values were lower than the k_q^S and a strong dependence on the reduction potential of quinone was observed. These facts are consistent with an electron-transfer quenching process:

$$(D^{-1})^* + Q \rightarrow (D^{\bullet} \ldots Q^{\bullet -1}) \rightarrow D^{\bullet} + Q^{\bullet -1} \qquad (1)$$

where $(D^{-1})^*$ and [Q] represent the excited triplet state of the dye and the quinone compound, respectively.

Table 2
Singlet and triplet quenching rate constants of resazurin and resorufin by *p*-benzoquinones in MeOH

Quinones (E_{red}/V)	$k_q^S/10^9$ M^{-1} s^{-1}		$k_q^T/10^9$ M^{-1} s^{-1}	
	Resazurin	Resorufin	Resazurin	Resorufin
DQ (−0.63)	7.2	8.0	0.025	1.5
DMQ (−0.51)	7.5	8.6	0.48	2.3
MQ (−0.45)	8.3	10.0	0.54	3.5
BQ (−0.40)	8.5	13.0	0.79	2.8
ClQ (−0.37)	–	–	1.33	2.5

Table 3
Triplet quenching rate constants of resazurin and resorufin by *p*-benzoquinones in water at pH 10

Quinones (E_{red}/V)	$k_q^T/10^9$ M^{-1} s^{-1}	
	Resazurin	Resorufin
DQ (−0.42)	2.5	2.8
DMQ (−0.29)	5.2	4.9
MQ (−0.22)	4.5	4.1
BQ (−0.14)	4.1	2.9
ClQ (−0.04)	–	3.0

On the other hand, the values of k_q^T for resorufin in MeOH are nearly constant and independent of the reduction potential of the quinone (Table 2), but lower than the diffusional limit expected for this solvent. This behavior was observed in other systems [24,25]. Also, from Table 2 it is evident that in MeOH the values of the triplet quenching rate constants are higher for resorufin than for resazurin. These results can be explained considering the probable differences in the triplet energy of the dyes. Higher triplet energy might be anticipated for resorufin from the differences in singlet energy according to the ground-state absorption spectrum and assuming a similar and small singlet–triplet energy difference for the dyes. The high values and the lack of dependence on reduction potential for resorufin might be due to a quenching mechanism involving the formation of an exciplex.

In water (Table 3), the k_q^T values are close to the diffusion limit (5×10^9 M^{-1} s^{-1}) for both dyes. In this solvent the reduction potential of the quinones is much smaller than in MeOH or acetonitrile [26] and this might be the explanation for the relatively higher values of the rate constants. This is also consistent with the lower value observed for DQ, the quencher with the higher potential. Unfortunately, values for the driving force of the electron-transfer processes (Reaction (1)) could not be estimated due to the impossibility of measuring the potential of resazurin (for resorufin the redox potentials are 0.49 V and 0.57 V, in water and MeOH, respectively), and the lack of data for the triplet energy values of both dyes. Consequently, the electron-transfer nature was further confirmed by the transient absorption spectra.

3.2. Transient absorption spectra

3.2.1. Resazurin

In a previous work [10], we determined the transient absorption spectrum of argon saturated aqueous solution of resazurin at pH 10 irradiated at 532 nm. The spectrum presents two broad bands, one in the 650–800 nm region and the other in the region around 400 nm. Fig. 2 shows the transient absorption spectrum obtained in MeOH. The spectrum is similar to that in

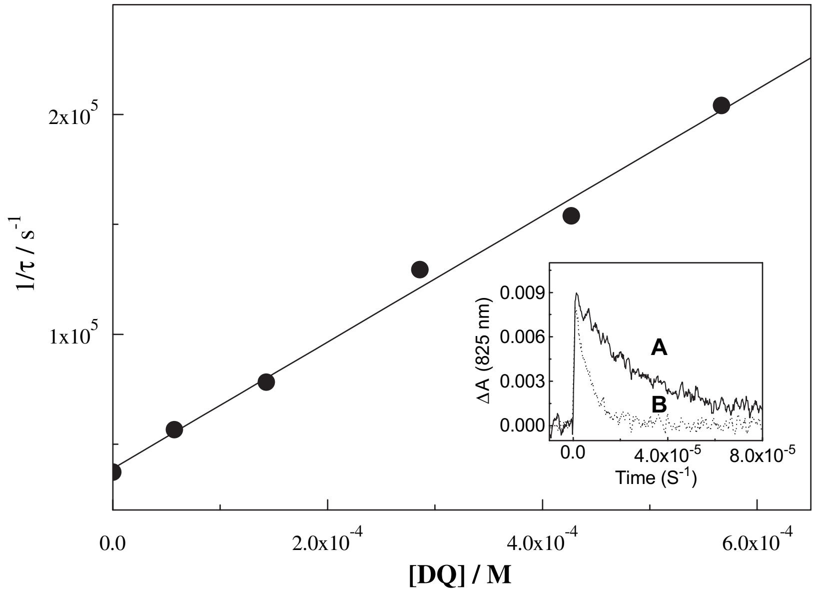

Fig. 1. Stern–Volmer plots of the triplet quenching of resazurin by duroquinone. Inset: (A) decay at 825 nm of resazurin in water at pH 10 and (B) decay in the presence of duroquinone 4.2×10^{-4} M.

aqueous solution and can be ascribed to the triplet state of the dye.

The negative absorption (500–620 nm) and the valley at 379 nm indicate the depletion of the ground state. The inset in Fig. 2 shows that the signal at 825 nm decays with a clear first-order kinetics with lifetime of 30 μs. The decay of the signal at 400 nm is similar to that at 825 nm, but a long-lived absorption remains.

There was an important autoquenching in this dye, due to the reaction between the triplet and ground state to give the semioxidized and semireduced species of the dye [11]; the value of the rate constant was $4.3 \times 10^{8}\ M^{-1}\ s^{-1}$. The spectrum of the dye at longer time in Fig. 2 can be assigned to the semireduced species with absorption at 400 nm while the band around 480 nm can be ascribed to the semioxidized species.

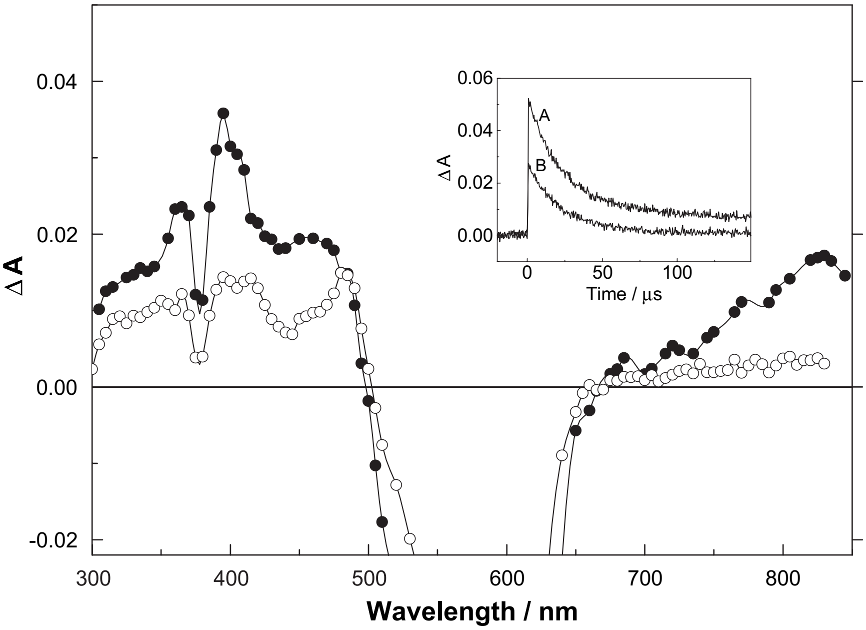

Fig. 2. Transient absorption spectra of resazurin at different time at 1 μs (●) and 50 μs (○) after the laser flash in MeOH. Inset: kinetics profile at 400 (A) and 825 nm (B).

The molar absorption coefficients of the triplet dye were determined in both solvents by the total depletion method. In both cases the negative absorption of the difference transient spectra matched the ground-state band. This is consistent with the lack of photoproduct formation under our conditions of laser experiments. Assuming that in the region of ground-state absorption the molar absorption coefficient of the triplet state is negligible, and using the measured absorption coefficient for the dye ground-state absorption, ε_T of $5400 \pm 1000\ M^{-1}\,cm^{-1}$ (825 nm) was obtained for the T–T absorption of resazurin in water at pH 10. The values obtained in MeOH were $4600 \pm 300\ M^{-1}\,cm^{-1}$ (825 nm).

In the presence of quinone (Fig. 3), at longtime the triplet quenching of resazurin was complete (there was no absorption in the region of 700–900 nm). The band at 480 nm that was attributed to the semioxidized form of the dye, increased in the presence of the quinone. The bands in the zone of 400 nm and 320 nm in Fig. 3 are due to the quinone anion radical [27]. This spectrum confirm the electron-transfer nature of the reaction.

3.2.2. Resorufin

Laser excitation at 532 nm of resorufin in MeOH resulted in the absorption spectra presented in Fig. 4. At short time intervals the spectrum exhibited two positive absorption bands in the 340–420 and 600–750 nm regions and a negative absorption that corresponds to the ground-state depletion. The signal at 700 nm decays with first-order kinetics. Meanwhile, the absorption at 420 nm increased with time at a rate similar to that of the decay of the species at 700 nm. This implies that the absorption at 420 nm is caused by species formed by the reduction of the species absorbing at 700 nm. The spectrum is similar to that in aqueous solution [16].

On the basis of the decay kinetics and sensitization experiments done in a previous work [11], the spectrum is assigned to the combined T–T absorption and the ground-state bleaching of resorufin. The first order rate constant measured at 700 nm was also dependent on the ground-state dye concentration. The self-quenching rate constant, evaluated from the linear plots of k_{obs} vs. the resorufin concentration, was $1.2 \times 10^9\ M^{-1}\,s^{-1}$.

As in the case of resazurin, the spectrum at 40 μs in Fig. 4 can be ascribed to a small residual absorption of the triplet state and to the semireduced and the semioxidized forms of the dye in the 350–440 nm region.

Using the same procedure as for resazurin, the following values were obtained for the triplet molar absorption coefficients of resorufin: ε_T $12\,500 \pm 500\ M^{-1}\,cm^{-1}$ (700 nm) and $15\,000 \pm 1000\ M^{-1}\,cm^{-1}$ (700 nm) for the T–T absorption of resorufin in MeOH and water at pH 10, respectively.

In the presence of quinone (Fig. 5), at longtime the triplet quenching of resorufin was complete (there was no absorption in the region of 600–750 nm). The band at 420 nm was previously attributed to the semioxidized form of the dye [11], and it increased in the presence of the electron acceptor. The band in the zone of 400 nm is due to the quinone anion radical [27]. At longer times a new band absorption appears probably due to a reaction of the semioxidized form of the dye with the quinone. This reaction is only present for resorufin and it needs further investigation.

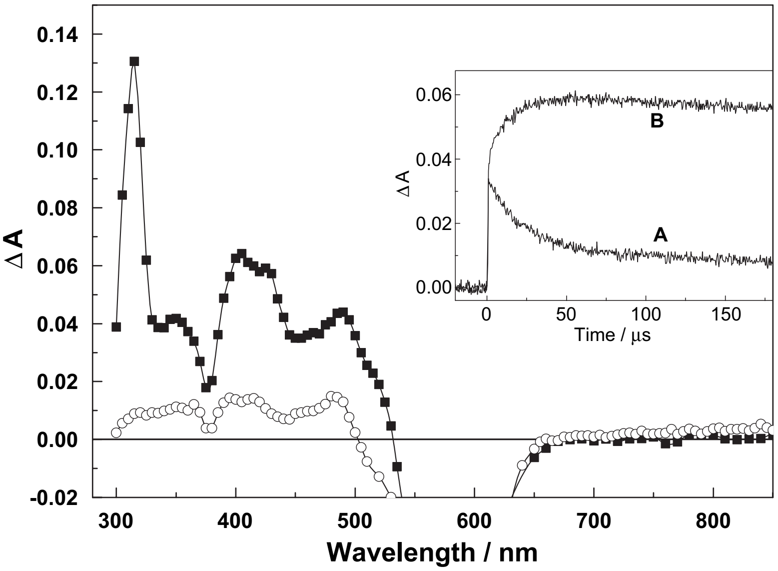

Fig. 3. Transient absorption resazurin in the absence (○) and presence (■) of *p*-benzoquinone 1×10^{-3} M 50 μs after laser flash in MeOH. Inset: kinetics profile at 420 nm in the absence (A) and in the presence (B) of *p*-benzoquinone at 420 nm.

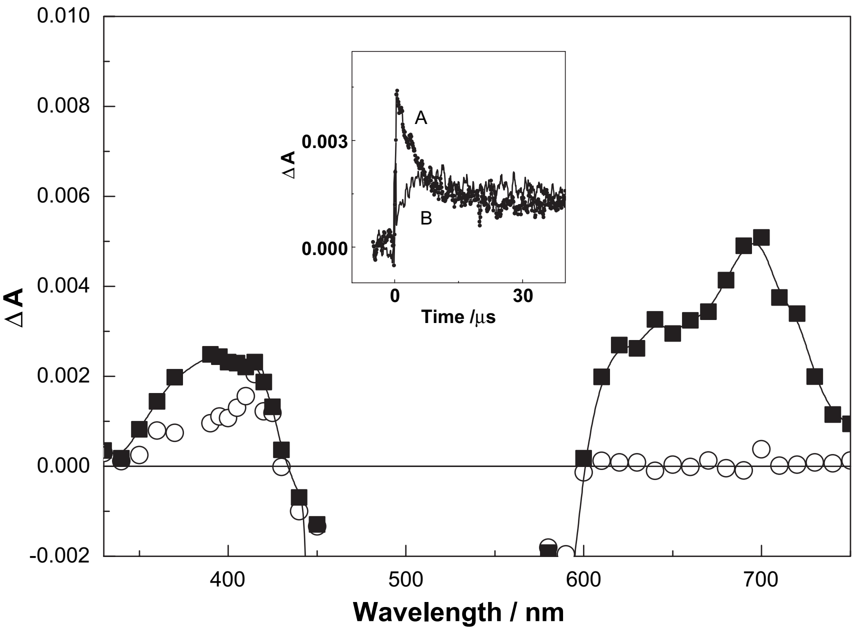

Fig. 4. Transient absorption spectra of resorufin at 5 μs (■) and 40 μs (○) after the laser flash in MeOH. Inset: kinetics profile at 700 (A) and 420 nm (B).

In summary, photophysical and photochemical properties of the phenoxazin-3-one dyes, resazurin and resorufin, have been quantitatively investigated in MeOH and aqueous solution at pH 10, based on the measurements of absorption and fluorescence spectra, fluorescence lifetimes and transient absorption. In water, both dyes are fluorescent only in their anionic forms at pH above 7.0. Resorufin presents a very high fluorescence quantum yield in neutral and basic media.

Singlet and triplet excited-states of these dyes are quenched by quinones. The quenching rate constants correlate with the reduction potential of the quinones in the nondiffusional zone. By laser flash photolysis we can observe the ion radicals and all evidence allows us to

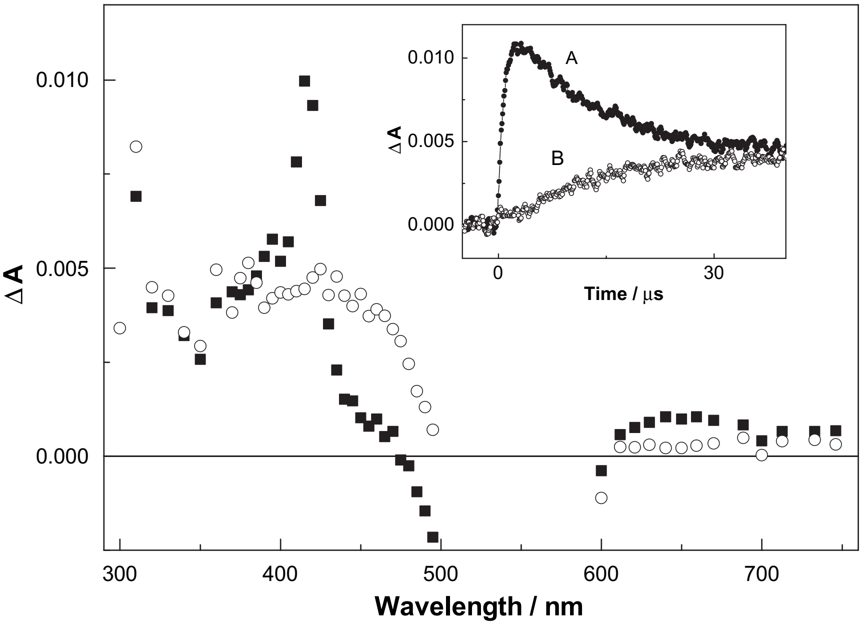

Fig. 5. Transient absorption resorufin in the presence of *p*-benzoquinone 1×10^{-3} M at 5 (■) and 40 μs (○) after laser flash in MeOH. Inset: kinetics profile at 420 (A) and 450 nm (B).

suppose that the quenching proceeds through an electron-transfer mechanism.

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