ELSEVIER

Journal of Photochemistry and Photobiology
A: Chemistry 87 (1995) 243–247

Journal of PHOTOCHEMISTRY AND PHOTOBIOLOGY A:CHEMISTRY

Photochemical mimetics of chromogenic catalymetric reactions of arylamines in water solutions

Vladimir M. Mekler, Olga V. Belonogova, Victor I. Nikulin
Institute of Chemical Physics, Chernogolovka, Moscow Region 142432, Russia

Received 8 June 1994; accepted 9 November 1994

Abstract

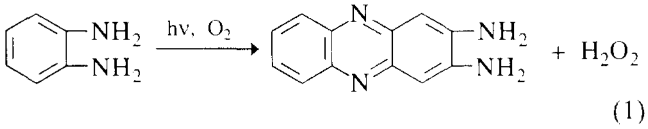
The photosensitized formation of coloured products from arylamines, which are used as chromogenic reagents in catalymetric oxidative reactions, and from some 3,4-substituted derivatives of *o*-phenylenediamine (*o*-PD) was investigated in aqueous buffer solutions. The absorption spectra of the photochemically formed coloured products were similar to those obtained by H_2O_2 oxidation. The photosensitized reactions of the compounds studied were observed in the presence of dioxygen only and were accompanied by H_2O_2 formation. It was found that the photo-oxidative colouration of solutions of all the catalymetric reagents studied could be sensitized by photoactive dyes, whereas autosensitized colouration was observed only for *o*-PD derivatives and *o*-aminophenol. The largest rate constants for the dye-sensitized and autosensitized reactions were found for 3,3′,5,5′-tetramethylbenzidine and 3-trifluoromethyl-1,2-phenylenediamine respectively. With regard to the application of the autosensitized formation of 2,3-diaminophenazine from *o*-PD to analytical studies, the conditions required to enhance the quantum yield of this reaction were evaluated.

Keywords: Mimetics; Chromogenic catalymetric reactions; Arylamines; Photo-oxidation; Sensitization

1. Introduction

The formation of coloured products from colourless arylamines by oxidative reaction in water solution is widely used as a catalymetric reaction in analytical chemistry and biomedical assays. The catalysis of the H_2O_2-mediated oxidation of chromogenic arylamines by transition metal ions, I^- and Br^- is used for the assay of these ions [1]. A very efficient catalyst of the oxidation of some arylamines by H_2O_2 is enzyme peroxidase. The application of *o*-phenylenediamine (*o*-PD) and 3,3′,5,5′-tetramethylbenzidine (TMB) as substrates of horseradish peroxidase (HRP) enables high sensitivity to be obtained in enzyme-linked immunosorbent assay (ELISA) [2,3].

Several investigations of the analytical applications of the photosensitized chromogenic reactions of arylamines have been reported [4–10]. The riboflavin-sensitized photo-oxidation of *o*-dianisidine was used to develop sensitive techniques of detection of manganese and superoxide dismutase activity [4,5]. The photo-oxidation of *o*-aminophenol (*o*-AP) in the presence of 2,6-anthrachinone disulphonic acid was used to assay molybdenum and tungsten [6]. A recent investigation of the photochemistry of *o*-PD solutions has shown that photo-oxidative coupling of *o*-PD to 2,3-diaminophenazine (DAP) (Eq. (1)) is initiated by the illumination of *o*-PD, photoactive dyes and DAP [7].



(1)

The DAP-sensitized *o*-PD oxidation is an autosensitized reaction and the dependence of the DAP concentration on the illumination time is close to exponential. Conjugation of the autocatalytic *o*-PD photo-oxidation with conventional *o*-PD catalymetric reactions has been proposed for the development of sensitive analytical assays [7,8]. Prompt colouration of an analysed solution and avoidance of the problem of sensitizer bleaching are the advantages of this approach. It is of interest to determine whether other similar chromogenic reactions are autosensitizable.

In this paper, the chromogenic photo-oxidative reactions of arylamines and some 3,4-substituted derivatives of *o*-PD were studied. The structures of the arylamines are shown in Fig. 1. The photochemical formation of coloured products from the colourless arylamines was observed by UV illumination of the arylamines (self-sensitized oxidation) and by

1010-6030/95/$09.50 © 1995 Elsevier Science S.A. All rights reserved
SSDI 1010-6030(94)03998-4

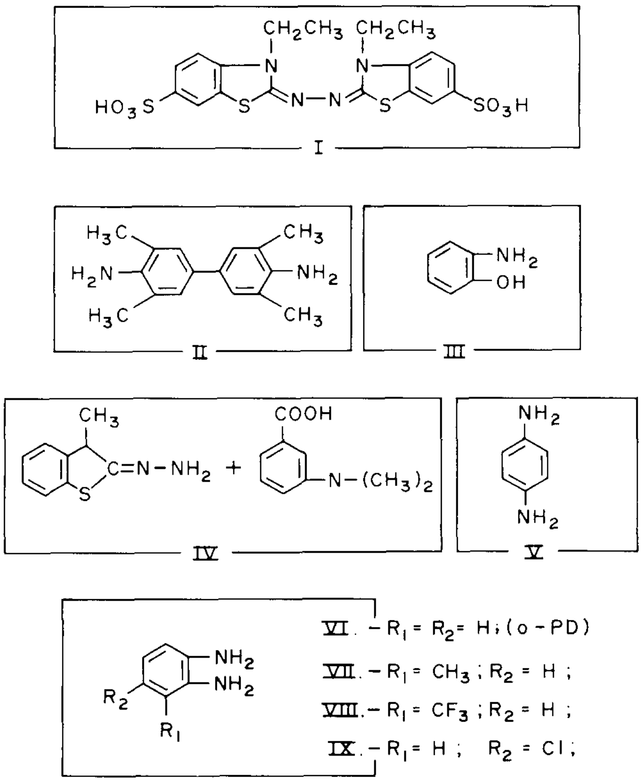


Fig. 1. Structures of the arylamines studied.

illumination with visible light of photoactive dyes (dye-sensitized oxidation) or the coloured oxidation products (autosensitized oxidation). Dye-sensitized chromogenic catalymetric reactions were observed for all the compounds studied, whereas autosensitized colouration was found only for *o*-PD derivatives and *o*-AP. With regard to the application of the autosensitized reaction (Eq. (1)) in a photochemically amplified ELISA [8], the conditions required to enhance the quantum yield of DAP formation (ϕ_{DAP}) were evaluated.

2. Experimental details

2.1. Reagents

Eosin, erythrosin, rose bengal, cyanosin, methylene blue, citric acid and HRP (activity, 250 U (mg protein)$^{-1}$) were purchased from Serva. Triton X-100 was purchased from Merck. 2,2′-Azinobis(3-ethylbenzthiazoline)sulphonic acid (**I**), *o*-AP (**III**) and *p*-phenylenediamine (**V**) were purchased from Fluka. 3,3′,5,5′-Tetramethylbenzidine (**II**), 3,4-diaminotoluene and 2,3-diaminotoluene (**VII**) were purchased from Aldrich. Components of the Ngo–Lenhoff reagent (**IV**), 2-methyl-2-benzothiazolinone hydrazone (MBTH) and 3-dimethylaminobenzoic acid (DMBA), were purchased from Fluka. *o*-PD (**VI**), *o*-AP and 4-chloro-1,2-phenylenediamine (**IX**) (Reakhim) were purified by vacuum sublimation. 3-Acetaminobenzotrifluoride was a gift from Dr. A. Sipyagin (Institute of Chemical Physics, Chernogolovka).

3-Trifluoromethyl-1,2-phenylenediamine (**VIII**) and 4-trifluoromethyl-1,2-phenylenediamine were obtained as described in Ref. [11] by nitration of 3-acetaminobenzotrifluoride, resolution of the isomers from the hydrolysed reaction mixture and reduction of each isomer with stannous chloride dihydrate. The compounds were purified by silica gel column chromatography. The arylamines studied are shown in Fig. 1. Solutions of the arylamines were used immediately after dissolution.

2.2. Instruments

Absorption spectra were recorded using a Specord-M40 UV–visible spectrophotometer in a square 1 cm cuvette. Fluorescence spectra were obtained with an Aminco-Bowman spectrofluorometer.

The irradiation source was a 1000 W mercury lamp. DAP and the products of oxidation of **III** and **VII–IX** were excited at 436 nm; erythrosin and cyanosin were excited at 546 nm. These lines of the mercury lamp were isolated using glass filters (LOMO, St. Petersburg) and interference filters (Carl Zeiss, Jena). Light intensities (N) were measured with a spectroradiometer. All measurements, unless otherwise stated, were made at room temperature (19 ± 1 °C). Deoxygenation of the solutions was carried out by pumping and N_2 bubbling. The DAP extinction coefficient of 2.1×10^4 M^{-1} cm^{-1} at 440 nm [7] was used.

2.3. Measurements of the rate constants of the photochemical reactions

The rate constants of the photosensitized reactions were measured in the following solutions recommended for HRP-mediated reactions [2]: 2 mM solution of **I** in 0.1 M acetate buffer (pH 4.2); **II**, predissolved in dimethylsulphoxide, was dissolved in sodium acetate/citrate buffer (0.2 M, pH 4) (final concentration of **II**, 1 mM); Ngo–Lenhoff reagent (**IV**) was composed of 65 μM MBTH and 3.3 mM DMBA in 0.1 M citrate/phosphate buffer (pH 7). The reactions of the other arylamines were performed in 0.01–0.1 M citrate buffer (pH 5) at arylamine concentrations of 4×10^{-3} M. Unless otherwise stated, the concentration of citrate buffer was 0.02 M. The rate constants of the photosensitized reactions were calculated from the kinetics of formation of the coloured products within the range of D values of 0.02–0.1. The dye-sensitized reactions were performed using 1 μM erythrosin as sensitizer.

2.4. Measurement of kinetic isotope effect

Measurements of the kinetic isotope effect on the erythrosin-sensitized reactions of **I–VI** were carried out in 0.02 M citrate buffer (pH 5) and in D_2O solution at an erythrosin concentration of 10 μM. The concentrations of MBTH and DMBA were 0.05 mM and 0.5 mM respectively. The concentration of **I–III**, **V** and **VI** was equal to 0.2 mM.

2.5. Comparison of the yields of the coloured products from the photochemical and H_2O_2-mediated oxidative reactions

A comparison of the yields of the coloured products from the photochemical and H_2O_2-mediated (HRP-catalysed) reactions was carried out spectrophotometrically by measuring the peak optical density (D) of the products. Cyanosin (10 μM) was used as the sensitizer in these experiments. The photochemical reactions were performed with stirring.

The buffer compositions used for the photochemical and H_2O_2-mediated reactions were the same as given in Section 2.3. The arylamine concentrations were the same as described in Section 2.4.

3. Results and discussion

3.1. Photo-oxidation of arylamines. Spectral and photochemical characteristics

Photosensitized colouration of solutions of the arylamines **I–IX** was observed in the presence of dioxygen only and was accompanied by H_2O_2 formation, as in the case of *o*-PD photo-oxidation [7]. This shows that the photosensitized colouration results from the oxidation of the arylamines by dioxygen. The HRP-mediated reactions of arylamines were used as a test for the detection of H_2O_2 formation. Therefore some HRP (0.1 U ml^{-1}) was added to an arylamine solution in which coloured products had been formed by irradiation. The addition of HRP caused an increase in the optical density of the solution by 1.7–2.1 times.

Self-sensitized oxidation was found for **II**, **IV** and **VI–IX**; particularly large rates of self-sensitized colouration were observed for solutions of **II** and **IV**. Xanthene dyes (eosin, erythrosin, cyanosin, rose bengal) and methylene blue sensitized the photo- oxidation of all the arylamines shown in Fig. 1. Taking into consideration the previously described dye-sensitized oxidation of *o*-dianisidine [4], it may be concluded that the dye-sensitized formation of coloured products is typical for the arylamines used in chromogenic catalymetric assays. However, autosensitized oxidation was observed for *o*-PD derivatives only and, to some degree, for *o*-AP. The kinetics of autosensitized colouration of *o*-AP were exponential at low D only. In the region $D > 0.1$, they were linear rather than exponential. The absorption spectra of the coloured products of self-, auto- and dye-sensitized reactions were similar and the same as the HRP-mediated oxidation spectrum for each of the arylamines **I–IX**. The photochemically obtained coloured products of **II**, and in particular those of **IX**, were unstable and the solutions were essentially bleached within 30 min. Fig. 2 shows the absorption spectra of the products of the *o*-PD derivatives **VII–IX** obtained by autosensitized oxidation. The absorption spectra of the coloured products of oxidation of the arylamines **I–VI** were analogous to those described in Refs. [2,12]. Some enhancement of the peak D values of **VII** and **VIII** solutions was observed after illumination was stopped, analogous to the post- illuminated changes in the spectra of *o*-PD solutions [13]. The fluorescence spectra of the coloured products of **VII–IX** photo-oxidation were similar to those of DAP [14] in their shape and large Stokes shift.

Prolonged irradiation of a sensitizer in the presence of **I–IX**, and prolonged autosensitized reaction, caused the peak D value in the visible region to increase monotonically until a maximum value was reached. Further exposure led to a decrease in the peak D value, while the absorption in the UV increased, probably due to the photodestruction of coloured products. To

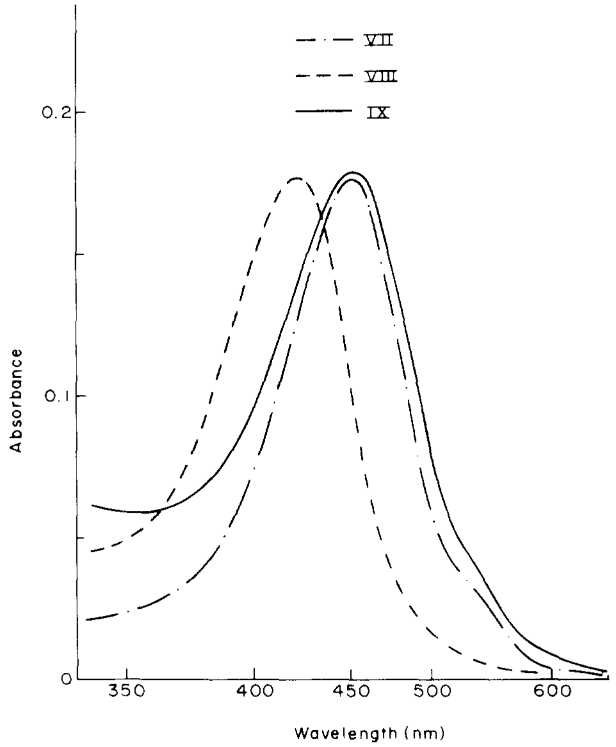


Fig. 2. Absorption spectra of the coloured products of autosensitized oxidation of arylamines **VII–IX** in 0.02 M citrate buffer (pH 5). The spectra were obtained by illumination of 4×10^{-3} M solutions of **VII–IX** at 436 nm.

compare the yields of the coloured products from the photochemical and H_2O_2-mediated oxidations, the maximum D values obtained from the photochemical (autosensitized and cyanosin-sensitized) and HRP-catalysed oxidations of **I–IX** were measured. The ratios of these D values are given in Table 1.

A comparison of the rate constants of the photosensitized colouration of the arylamines **I–IX** with those of *o*-PD is given in Table 2. As regards these data, it should be noted that the main coloured products of the oxidation of **I** and **II** result from one- and two-electron oxidation of the compounds, whereas the formation of stable coloured products from **III–VI** requires the withdrawal of four electrons from the initial arylamine molecules [2,12].

Catalymetric arylamines are relatively readily oxidized. The formation of coloured products was observed immediately after the addition of 2×10^{-4} M $K_3Fe(CN)_6$ ($E°=0.36$ V) to solutions of **I–VI**. The fast kinetics of oxidation of **III**, **V** and **VI** by $K_3Fe(CN)_6$ were studied by Nogami et al. [12]. The dyes used in this work are known to be effective photogenerators of singlet oxygen. Since $^1O_2$ is a comparatively strong oxidant ($E°=0.65$ V [15]), it may be suggested that $^1O_2$ takes part in the dye-sensitized reactions studied. Support for this suggestion is obtained by the enhancement of the rate of the erythrosin-sensitized oxidation of **I–IV** and **VI** in a D_2O buffer solution compared with that in a similar H_2O solution (Table 2), which can be explained as a result of the increase in the $^1O_2$ lifetime in D_2O [16].

Table 1
Comparison of the yields of the coloured products from H_2O_2-mediated and photochemical oxidative reactions of arylamines

Compound	$D_{cy}/D_{H_2O_2}$	$D_a/D_{H_2O_2}$	λ (nm)
I	0.57	0	415
II	0.52	0	450
III	0.45	0.35	430
IV	0.82	0	590
V	0.53	0	555
VI	0.60	0.65	440
VII	0.48	0.45	445
VIII	0.64	0.65	420
IX	0.8	–[a]	450

[a] The measurement was not performed because of the instability of the coloured products of **IX**.

D_{cy}, D_a and $D_{H_2O_2}$ are the maximum D values of the coloured products obtained respectively by cyanosin-sensitized, autosensitized and H_2O_2-mediated oxidation of the arylamines measured at the peak wavelength (λ).

Table 2
Comparative kinetic characteristics of the photosensitized reactions of the arylamines

Compound	K_{er}/K_{er} (*o*-PD)	K_a/K_a (*o*-PD)	$K_{er}(D_2O)/K_{er}(H_2O)$
I	1.5	0	1.3
II	7.0	0	2.0
III	2.4	0.07	1.8
IV	2.9	0	2.6
V	0.6	0	0.7
VI	1 (0.008)	1 (0.0034)	3.3
VII	0.3	0.3	
VIII	1	2.4	
IX	0.4	0.5	

K_{er} and K_a are the rate constants of erythrosin-sensitized and autosensitized colouration respectively. The quantum yields of the autosensitized and erythrosin-sensitized *o*-PD reactions [7] are given in parentheses.

In addition to the compounds shown in Fig. 1, 4-methyl-1,2-phenylenediamine and 4-trifluoromethyl-1,2-phenylenediamine were tested. However, their solutions showed slow self-sensitized enhancement of absorption and no appreciable autosensitized or dye-sensitized colouration.

3.2. The dependence of the quantum yield of the autosensitized oxidation of o*-PD on the conditions of reaction*

A rather small value of $\phi_{DAP}=0.0034$ was found previously in a substrate solution of 4×10^{-3} M *o*-PD in 0.1 M phosphate/citrate buffer (pH 5) at 20 °C and $N=8\times10^{16}$ photons s^{-1} cm^{-2} [7]. This substrate solution is often used in HRP-mediated ELISA [2] and was used to develop photochemically amplified ELISA [8]. In this work, it was found that ϕ_{DAP} can be increased by making the following changes in the reaction conditions.

3.2.1. Influence of buffer concentration

A tenfold dilution of 0.1 M citrate or phosphate/citrate buffer solutions results in a 2.1-fold increase in ϕ_{DAP}. Further dilution gives no appreciable change in ϕ_{DAP}. The addition of NaCl to the 0.01 M buffer solution decreases the ϕ_{DAP} value. On the other hand, for erythrosin-sensitized *o*-PD oxidation, less than a 10% change in K_{er} was observed with similar variations of the buffer composition. It was also found that the enhancement of ϕ_{DAP} was accompanied by a closely matched enhancement of DAP fluorescence intensity. These results suggest that the effect of buffer concentration on ϕ_{DAP} can be explained by the dependence of the quantum yields of the radiationless transitions of DAP excited states on the ionic strength of the solution rather than by known salt effects on photoinduced electron transfer [17].

3.2.2. Addition of Triton X-100

Micelles of Triton X-100 and other non-ionic surfactants absorb DAP in water solutions, resulting in increased DAP fluorescence intensity [14]. A 3.7-fold enhancement of ϕ_{DAP} was observed on addition of 2% Triton X-100 (v/v) to 0.05 M citrate buffer solutions.

Inhibition of autosensitized *o*-PD oxidation was observed in the presence of anionic surfactants which effectively bind DAP.

3.2.3. Addition of methyl viologen

The use of methyl viologen enables large quantum yields of radical photogeneration to be obtained in some photochemical reactions [18]. Therefore methyl viologen was used as an intermediate electron carrier to enhance ϕ_{DAP}. The addition of 10^{-3} M methyl viologen to the solution used in Ref. [7] gave a 1.9-fold increase in ϕ_{DAP}.

3.2.4. Elevation of temperature

As shown in Fig. 3, the rate of the autosensitized reaction (Eq. (1)) is increased with increasing temperature. The initial rate of DAP formation at 50 °C was 3.2-fold larger than that at 15 °C. The kinetics of the reaction become essentially non-exponential in the range of large optical densities at high temperature.

3.2.5. Decreasing illumination intensity

Decreasing illumination intensity results in some enhancement of ϕ_{DAP}. When N is decreased from 8×10^{16} to 4×10^{15} photons $\mathrm{s^{-1}\ cm^{-2}}$, the ϕ_{DAP} values increase by 1.7 and 1.4 times in 0.1 M and 0.02 M citrate buffer solutions respectively.

3.2.6. Combination of effects

The combination of the above effects produces a diversified influence on ϕ_{DAP}. For example, the effect of decreasing illumination intensity decreases on dilution of the buffer. On the other hand, the joint effect of decreasing the buffer concentration and increasing the temperature is close to the product of the respective individual effects. A ϕ_{DAP} value of 0.019 was measured in 0.025 M citrate buffer solution (pH 5) at 40 °C and $N=2\times10^{15}$ photons $\mathrm{s^{-1}\ cm^{-2}}$, which is 5.6-fold larger than that observed in Ref. [7]. It may be noted that the latter buffer is suitable for HRP-mediated ELISA.

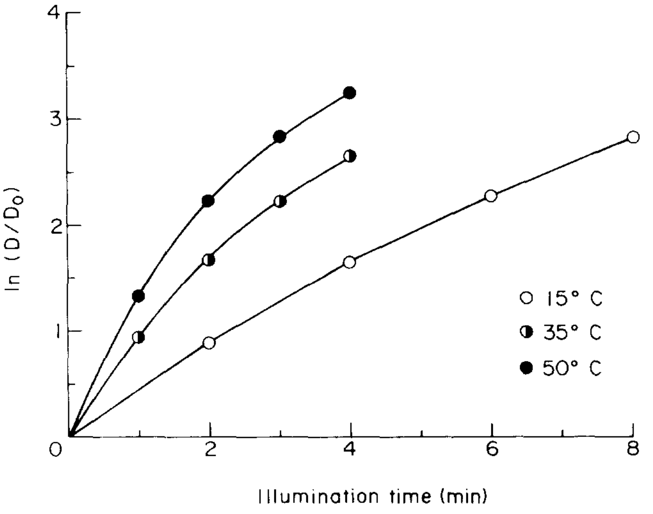


Fig. 3. Temperature dependence of the kinetics of *o*-PD autosensitized oxidation. Measurements were carried out in 0.02 M citrate buffer (pH 5), [*o*-PD] = 4×10^{-3} M. Optical densities (D) were measured at 440 nm. The initial optical density (D_0) was 0.02.

4. Conclusions

The results show that arylamines, commonly used as catalymetric reagents, are readily photo-oxidized in the presence of sensitizers; this should be taken into account when developing assays. On the other hand, autosensitized reactions were observed only for *o*-PD derivatives and for *o*-AP. The quantum yield of the autosensitized reaction (Eq. (1)) is affected by various parameters and can be enhanced considerably compared with the value obtained previously.

Acknowledgement

This work was supported by a grant from the Russian Foundation for Fundamental Sciences (93-04-7208).

References

[1] M. Kopanica and V. Stara, Kinetic methods in chemical analysis, in C.L. Wilson and D.W. Wilson (eds.), *Comprehensive Analytical Chemistry*, Vol. 18, Elsevier, Amsterdam, 1983.
[2] B. Porstmann and T. Porstmann, in T.T. Ngo (ed.), *Nonisotopic Immunoassay*, Plenum, New York, 1988, p. 57.
[3] T. Porstmann and S.T. Kiessig, *J. Immunol. Methods, 150* (1992) 5.
[4] H.P. Misra and I. Fridovich, *Arch. Biochem. Biophys., 183* (1977) 511.
[5] W.F. Beyer, Jr. and I. Fridovich, *Anal. Biochem., 70* (1988) 512.
[6] Y.K. Shazzo and I.P. Charlamov, *Zavod. Lab., 49* (1983) 17.
[7] V.M. Mekler and S.M. Bystryak, *J. Photochem. Photobiol. A: Chem., 65* (1992) 391.
[8] S.M. Bystryak and V.M. Mekler, *Anal. Biochem., 202* (1992) 390.
[9] M. Kaneko, N. Sagamura and A. Jamada, *Sci. Papers Inst. Phys. Chem. Res. (Jpn.), 72* (1978) 137.
[10] U. Nickel, Y.H. Chen, S. Shneider, M.I. Silva, H.D. Burrows and S.J. Formosinho, *J. Phys. Chem., 98* (1994) 2883.
[11] E. Pouterman and A. Girardet, *Helv. Chim. Acta, 30* (1947) 107.
[12] T. Nogami, T. Hishida, M. Jamada, H. Mikawa and A. Shirota, *Bull. Chem. Soc. Jpn., 48* (1975) 3709.
[13] V.M. Mekler and S.M. Bystryak, *Khim. Phys., 10* (1991) 1469.
[14] V.M. Mekler and S.M. Bystryak, *Anal. Chim. Acta, 264* (1992) 359.
[15] W.H. Koppenol, *Nature, 262* (1976) 420.
[16] D.R. Kearns, in H.H. Wasserman and R.W. Murray (eds.), *Singlet Oxygen*, Academic Press, New York, 1979, p. 115.
[17] J. Santamaria, in M.A. Fox and M. Chanon (eds.), *Photoinduced Electron Transfer*, Part B, Elsevier, Amsterdam, 1988, p. 483.
[18] K. Mandal and M.Z. Hoffman, *J. Phys. Chem., 88* (1984) 5632.