ELSEVIER

Journal of Photochemistry and Photobiology
A: Chemistry 89 (1995) 45–51

Journal of
PHOTOCHEMISTRY
AND
PHOTOBIOLOGY
A:CHEMISTRY

Spectroscopic study on the photoreduction of hypocrellin A: generation of semiquinone radical anion and hydroquinone

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Received 11 October 1994; accepted 10 January 1995

Abstract

Hypocrellin A (HA) is an efficient phototherapeutic agent. Illumination of HA in dimethylsulphoxide (DMSO) or DMSO–buffer (1:1 by volume, pH > 6.7) generated a strong electron paramagnetic resonance (EPR) signal. This EPR signal was intensified in the presence of reductants. The EPR spectrum obtained was assigned to the semiquinone radical anion of HA ($HA^{\cdot -}$) based on a series of experimental results. Decay of $HA^{\cdot -}$, attributed to a radical–radical reaction, follows second-order kinetics. In acidic DMSO–buffer (1:1 by volume) solution, no EPR signal of $HA^{\cdot -}$ was detected in the absence or presence of reductant. This was explained by a fast disproportionation of radicals, facilitated by protonation of radicals. The spectrophotometric measurements indicated that on illumination HA was directly reduced to its two-electron reduction product, i.e. hydroquinone, in acidic solution. The absorption maximum of the hydroquinone of HA which is at 496 nm at pH 5.8 shifts bathochromically with increase in pH of the medium. In DMSO or DMSO–buffer (1:1 by volume, pH > 6.7) solutions the semiquinone radical anion of HA was also observed spectrophotometrically. The absorption maximum of $HA^{\cdot -}$ is at around 628 nm. Strong intramolecular hydrogen bonding was considered to exist in the chromophore of $HA^{\cdot -}$.

Keywords: Hypocrellin A; Semiquinone radical anion; Hydroquinone; EPR spectra; Spectrophotometric measurements

1. Introduction

Hypocrellin A (HA), isolated from the parasitic fungus *Hypocrella bambusae* (*B. et Br.*) sacs [1], is an efficient phototherapeutic agent. It belongs to the perylenoquinone derivative group and exists as four equilibrated tautomeric isomers (T_1, T_2, T_3 and T_4) (Fig. 1) [2]. Since the acetylation of HA only gives the acetylated products of T_1 and T_2, T_1 and T_2 are considered to be the preferential tautomers [3,4].

In clinical trials, HA has produced promising results in the treatment of various skin diseases such as white lesions of vulva and vitiligo [5]. HA was found to kill Gram-positive bacteria effectively but not Gram-negative bacteria [1]. Also, damage to human erythrocyte membranes was observed when the cells were irradiated together with HA [6]. Recently, it has been shown that HA can kill tumour cells efficiently and can be accumulated selectively in cancer cells. Hela cells and S-180 solid tumour cells can be killed photodynamically, and the growth of mitochondrial ATPase and microsomal G-6-pase of hepatoma cells can be inhibited [7,8].

The chemistry, photochemistry and photophysics of HA have been studied extensively [3,4]. It has been shown that not only the active oxygen species ($^1O_2$, $O_2^{\cdot -}$ and $\dot{O}H$) but also the HA radicals ($HA^{\cdot -}$ and $HA^{\cdot +}$) participate in the photodynamic damage caused by HA [9,10]. The semiquinone radical anion ($HA^{\cdot -}$) was considered to be a key intermediate in the cytotoxic reactions, through its ability to generate toxic materials such as reactive oxygen species ($O_2^{\cdot -}$, H_2O_2 and $\dot{O}H$) and its reactions with the substrates [9,11,12]. The hydroquinone of HA might also play an important role in the cytotoxic reactions, since it can be oxidized to generate semiquinone radical anion and reactive oxygen species via one-electron oxidation. Therefore, a study on the photochemical and spectroscopic properties of the HA radical and its hydroquinone is of importance. In the present study, we report our results on the identification and the properties of the semiquinone radical anion and hydroquinone of HA.

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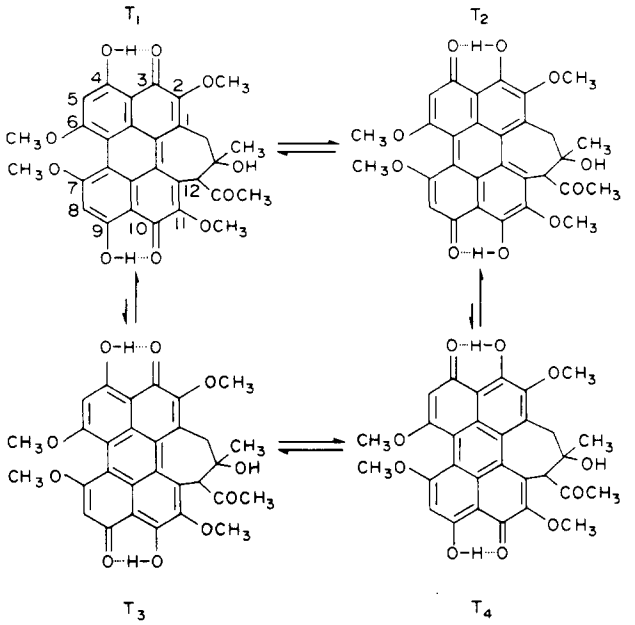

Fig. 1. Structure of HA.

2. Materials and methods

2.1. Materials

HA was isolated from the fungus sacs of *H. bambusae* and recrystallized twice from acetone before use. The reduced form of nicotinamide adenine dinucleotide (NADH), cystein, methionine, ascorbic acid, the reduced form of glutathione and superoxide dismutase (SOD) were purchased from Biotech Technology Corporation, Chinese Academy of Sciences. 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Aldrich Chemical Company and stored at −20 °C under argon. 1-benzyl-1,4-dihydronicotinamide (BNAH) was a gift from Dr. G. Deng. Other reagents used, all of analytical grade, were purchased from Beijing Chemical Plant. The required organic solvents of high purity were prepared by further purification of the commercial products. Solutions were freshly prepared before use and the pH was adjusted when necessary using phosphate buffer (for pH 5.2–8.3). The solutions were purged with argon, air or oxygen according to the experimental requirements.

2.2. Methods

UV–visible absorption spectra were recorded on an HP diode array spectrophotometer model 8451A. Photolysis experiments were performed under illumination of a medium pressure sodium lamp (400 W) on a "merry-go-round" apparatus. Light of wavelength below 470 nm was cut off by a long-pass filter, and the apparatus was immersed in running water in a thermostat at 20 °C. The solutions examined were put in glass cuvettes with a long neck. Argon was bubbled through to remove oxygen and then the cuvettes were sealed with a rubber stopper. After illumination, their absorption spectra were measured with a UV–visible spectrophotometer. Electron paramagnetic resonance (EPR) spectra were recorded at room temperature using a Varian E-1700BB spectrometer operating at 9.5 Hz (X band). Photoinduced EPR spectra were obtained from the samples introduced into a quartz capillary cell. The samples were illuminated directly in the cavity of the EPR spectrometer with a Shoeffel 1 kW Xe arc lamp. A filter was used to cut out light of wavelength below 470 nm.

3. Results and discussion

3.1. Electron paramagnetic resonance measurements of free radicals produced during photosensitization of hypocrellin A

Illumination of HA (1 mM) in an argon-gassed dimethylsulphoxide (DMSO) solution for 2 min generated the EPR spectrum shown in Fig. 2, spectrum A. The intensity of the signal increased rapidly during photolysis and decreased very slowly in the dark. When the sample was exposed to oxygen, the EPR signal was quenched. The EPR signal intensity of the HA radical depends also on the concentration of HA, illumination time and intensity. The concentration of HA exerts such a strong effect on the generation of the HA radical that the signal of the HA radical can be observed even in aerated DMSO at high concentration. The strong concentration effect indicates that the HA radical may be generated by self-electron transfer between the ground and excited species according to [13]

$$HA^* + HA \longrightarrow HA^{\cdot -} + HAa^{\cdot +} \quad (1)$$

Using HA as photosensitizer, a similar EPR signal has recently been obtained by Zang et al. [10], Wang et al. [11] and An et al. [12]. Wang et al. and An et al. ascribed it to the semiquinone radical anion of HA ($HA^{\cdot -}$). However, Zang et al. assigned it to the radical cation of HA ($HA^{\cdot +}$) based on the observation that in the presence of ascorbic acid or catechol the EPR spectrum was completely suppressed. In order to identify the EPR signal shown in Fig. 2, spectrum A, the following experiments were performed.

(1) HA (1 mM) in DMSO was illuminated in the presence of BNAH (5 mM), a typical reductant, for 30 s. The EPR spectrum obtained (Fig. 2, spectrum B) is very similar to that obtained in the absence of BNAH (Fig. 2, spectrum A). From Fig. 2 it can also be seen that the addition of BNAH intensifies this EPR signal significantly. This indicates the anionic character of the HA radical.

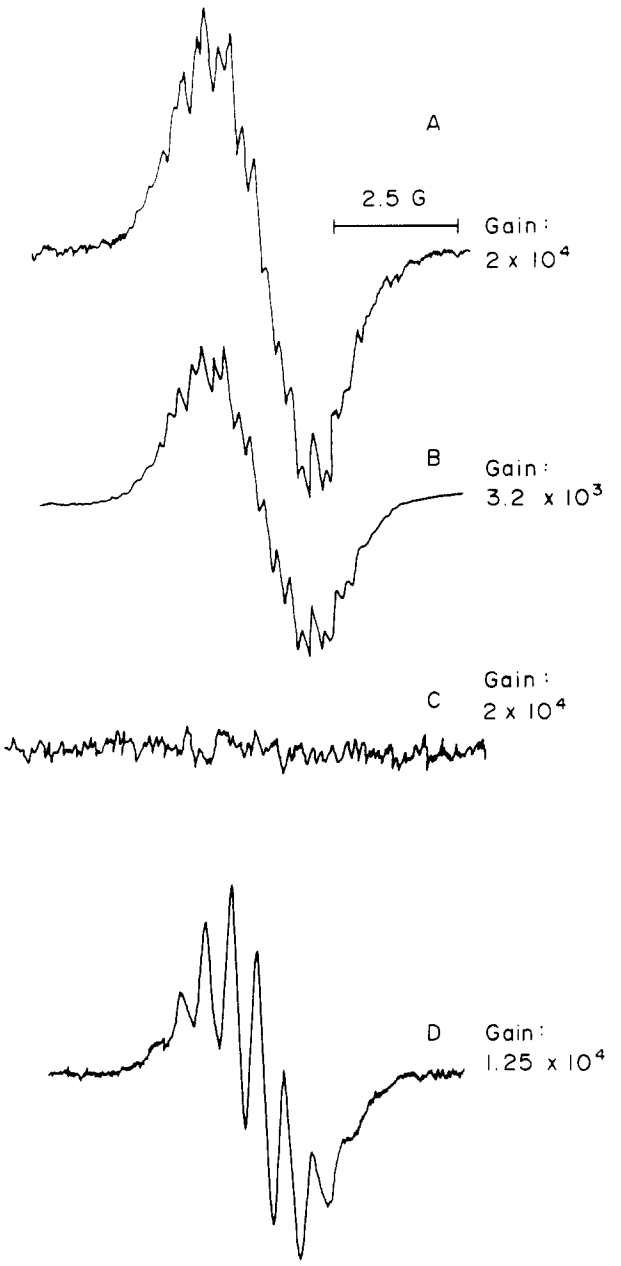

Fig. 2. Spectrum A, photoinduced EPR spectrum from the deaerated DMSO solution of HA (1 mM). Illumination was with light of wavelength above 470 nm for 2 min. Spectrum B, similar to spectrum A but in the presence of BNAH (5 mM) and illumination for 30 s. Spectrum C, same as spectrum B but oxygen was bubbled through the solution after illumination. Spectrum D, photoinduced EPR spectrum from the deaerated DMSO–buffer (1:1 by volume, pH 8.0) solution of HA (1 mM) and BNAH (5 mM) after illumination for 30 s. Instrumental settings: microwave power, 1 mW; modulation amplitude, 0.16 G; time constant, 0.128 s; receiver gain, 2×10^4 for spectrum A, 3.2×10^3 for spectrum B, 2×10^4 for spectrum C and 1.25×10^4 for spectrum D.

(2) A series of other reductive materials with different redox potentials were added to the HA solution. In all cases the EPR signals of the HA radical were intensified in comparison with that obtained without reductant after illumination for 2 min (Table 1). Table 1 also shows that the intensity increases sharply with the decrease in the redox potential of the reductive materials added. This further confirms the anionic nature of the HA radical.

(3) The EPR signal of the HA radical could be quenched significantly by oxygen. As shown in Fig. 2, spectrum C, the EPR signal disappeared completely when oxygen was bubbled through the solution of the HA radical. Furthermore, when DMPO and oxygen were involved in the solution of the HA radical, the EPR signal of the DMPO–superoxide radical adduct was detected immediately, accompanied by the disappearance of the EPR signal of the HA radical (Fig. 3, spectrum A) [14]. The spectroscopic parameters for Fig. 3A were determined to be $a^N = 12.80$ G, $a_\beta{}^H = 10.25$ G and $a_\gamma{}^H = 1.50$ G and are close to the values reported previously for the DMPO–superoxide radical adduct [15]. In the presence of the enzyme SOD the EPR signal of the DMPO–superoxide was quenched (Fig. 3, spectrum B).

(4) The kinetics of the decay of the radical was measured by recording the decrease in the amplitude of the EPR signal from its steady state level after illumination was stopped. The result is shown in Fig. 4(a). Fig. 4(a) shows that the HA radical decays according to second-order kinetics.

In accordance with these results, the spectra shown in Fig. 2 can be safely ascribed to the semiquinone radical anion of HA ($HA^{\cdot -}$) but not to the HA radical cation ($HA^{\cdot +}$). This ascription is further supported by a recent report [16] in which the EPR spectrum of $HA^{\cdot -}$ has been identified by spectral simulation. In the presence of the reductant, $HA^{\cdot -}$ may be generated by

$$HA^* + R \longrightarrow HA^{\cdot -} + R^{\cdot +} \quad (2)$$

As shown in Fig. 4(a), the termination of $HA^{\cdot -}$ is through a radical–radical annihilation reaction:

$$HA^{\cdot -} + HA^{\cdot -} \longrightarrow HA + HA^{2-} \quad (3)$$

The assignment of the HA radical to $HA^{\cdot +}$ in Ref. [10] was based on the observation that the EPR spectrum of the HA radical was completely suppressed when ascorbic acid (5 mM) was added to the solution of HA (1 mM). It is well known that semiquinone radical anion is quite basic and can be easily protonated to semiquinone radical in acid solution [12]. The protonation catalyses the disproportionation of the radical anion and the transfer of the second electron to give the two-electron reduction product and the starting quinone [17]. Under the experimental conditions employed in Ref. [10], the HA radical anion might be easily protonated since ascorbic acid is a stronger acid ($pK_1 = 4.04$) [18]. Therefore, the suppression of the EPR spectrum of the HA radical is probably due to the fast disproportionation of $HA^{\cdot -}$ (Eq. (3)) and/or

Table 1
Effect of reductive materials with different redox potentials on the intensity of the electron paramagnetic resonance signal of $HA^{\cdot -}$

Reductant	Relative intensity	$E_{R^{\cdot +}/R}$ (vs. normal hydrogen electrode) (V)
HA	1	1.10
Methionine	4	–
Glutathione	57	0.87
Cystein	127	0.63
NADH	277	0.28

The DMSO–buffer (1:1 by volume, pH 8.0) solution containing HA (1. mM) and reductant (5 mM) was illuminated for 2 min. Spectrometer settings: microwave power, 1 mW; modulation amplitude 1 G; time constant, 0.128 s.

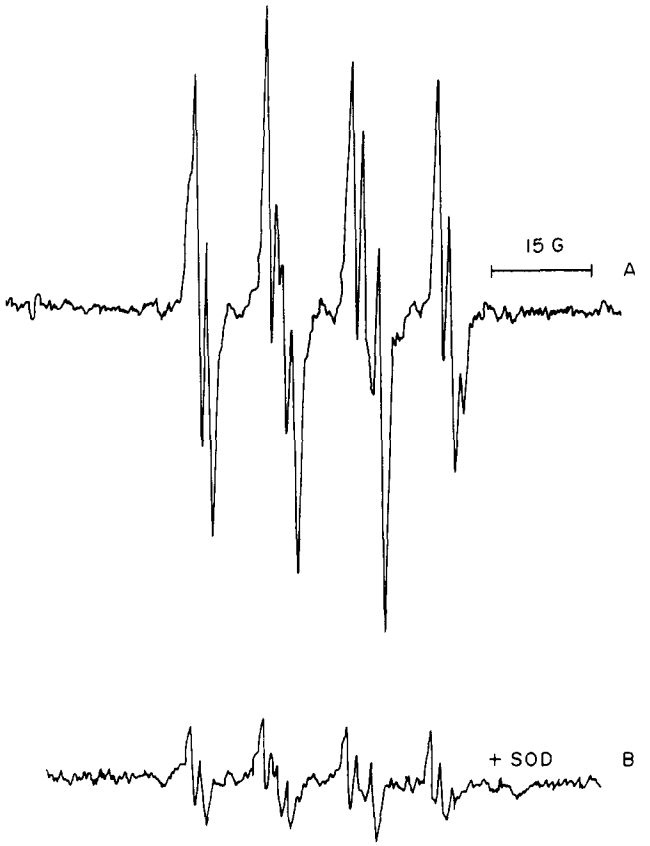

Fig. 3. Spectrum A, EPR spectrum of DMPO–superoxide radical adduct recorded after DMPO (45 mM) and oxygen were involved in a solution of $HA^{\cdot -}$. The solution of $HA^{\cdot -}$ was obtained by photoreduction of HA with BNAH as reductant. Spectrum B, same as spectrum A but in the presence of SOD. Spectral parameter settings: microwave power, 10 mW; time constant, 0.128 s; modulation amplitude, 1.0 G; receiver gain 1×10^4.

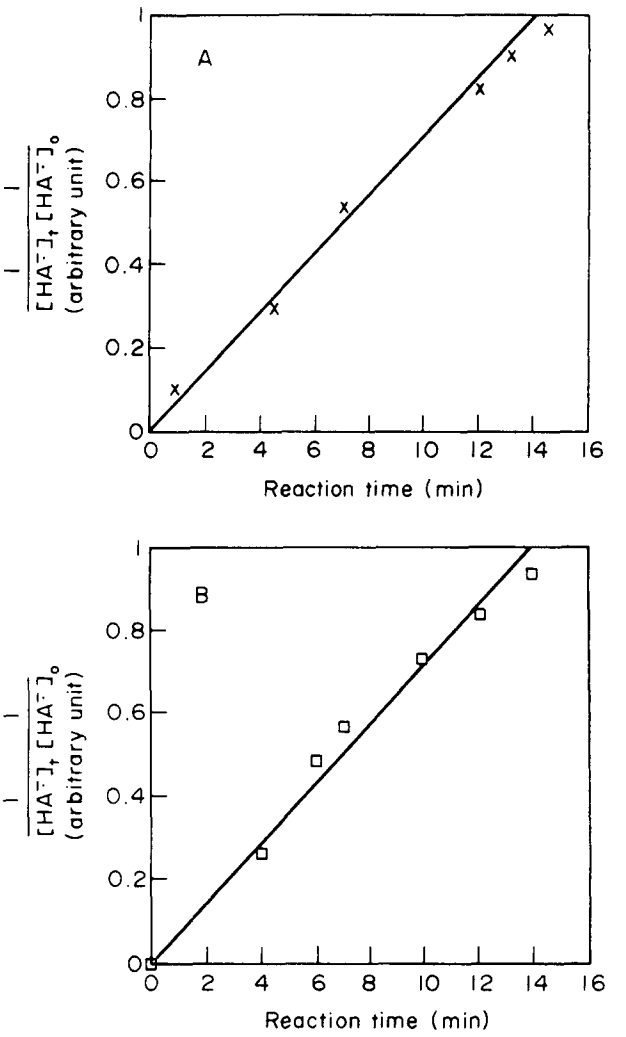

Fig. 4. Dependence of the reciprocal of the HA semiquinone radical anion concentration on the time of dark reaction in DMSO–buffer at pH 8.0: (a) measured from the decay of the amplitude of the EPR signal of $HA^{\cdot -}$; (b) measured from the decay of the optical density of $HA^{\cdot -}$ at 628 nm. $HA^{\cdot -}$ was generated by illumination of an argon-gassed DMSO–buffer (1:1 by volume, pH 8.0) of HA (0.5 mM) and ascorbic acid (8 mM).

the transfer of the second electron, facilitated by protonation of the radicals, but not to the reaction of ascorbic acid with $HA^{\cdot +}$. In agreement with this inference seems to be the observation that the efficiency of the photoinduced generation of the EPR signal from HA depends on the pH of the medium. While strong EPR spectra were produced in DMSO–buffer (1:1 by volume, pH > 6.7), no EPR signal was produced in DMSO–buffer (1:1 by volume, pH < 6.0) in the absence or presence of reductants (e.g. BNAH, NADH) but the colour of the illuminated mixture changed from red to pink–orange. This inference will be further

confirmed by spectrophotometric measurements (see Section 3.2). In fact, when adjusting the pH value of the deaerated solution of HA and ascorbic acid to pH 8.0 with NaOH, on illumination the EPR spectra of both the HA radical and $As^{\bullet -}$ were clearly observed (not shown) and they overlapped partially with each other. The amplitude of the EPR signal of the HA radical obtained was about 50 times larger than that obtained without ascorbic acid. This provides further support for the conclusion that the EPR spectrum generated on photosensitization of HA in DMSO or DMSO–buffer originates from the semiquinone radical anion of HA but not from the HA radical cation.

It was also observed that the hyperfine structure of the EPR spectrum of $HA^{\bullet -}$ was very susceptible to the alteration of solvent. As shown in Fig. 2, spectrum D, the EPR spectrum obtained in DMSO–H_2O (1:1 by volume) is much simpler than that in DMSO. In addition, the spectrum recorded in methanol is also different from that in DMSO, but similar to that in DMSO–H_2O (1:1 by volume). The effect of solvent on the EPR spectrum of the semiquinone radical anion might arise from a redistribution of π electron spin density resulting from localized complexes between the solvent and the oxygen atoms of the semiquinone ion [19,20]. Since HA is insoluble in H_2O, the attempt to study the EPR spectrum of $HA^{\bullet -}$ in H_2O was prevented. Further EPR studies on the effect of solvent and spectral simulation are currently in progress and will be reported in due course.

3.2. Spectrophotometric measurements of deoxygenated solutions of hypocrellin A and electron donor

Deaerated samples containing HA and BNAH or other reductants in DMSO and DMSO–buffer (1:1 by volume) at pH 5.8, 6.7, 7.3 and 8.0 were illuminated with light of wavelength above 470 nm at 5, 15 or 40 s intervals. The absorption spectrum was recorded after each exposure.

Photoreduction in acid solution (pH 5.8)

An example of the photoinduced optical changes for samples incubated at pH 5.8 is shown in Fig. 5. As shown in Fig. 5, on illumination, the absorption from HA (470 nm, 540 nm and 580 nm) and BNAH (356 nm) decreases and a new absorption band at 496 nm is formed. During the exposure the colour of the sample changes from red to pink–orange. There are two simultaneously appearing isosbestic points at 488 nm and 512 nm, indicating the presence of two light-absorbing forms of HA: the starting quinone, HA, and most likely hydroquinone, HAH^-. (Note that we will generally denote the reduced forms of HA as HAH^- and $HA^{\bullet -}$ without necessarily implying that the question of the protonation of these species has been finally resolved.)

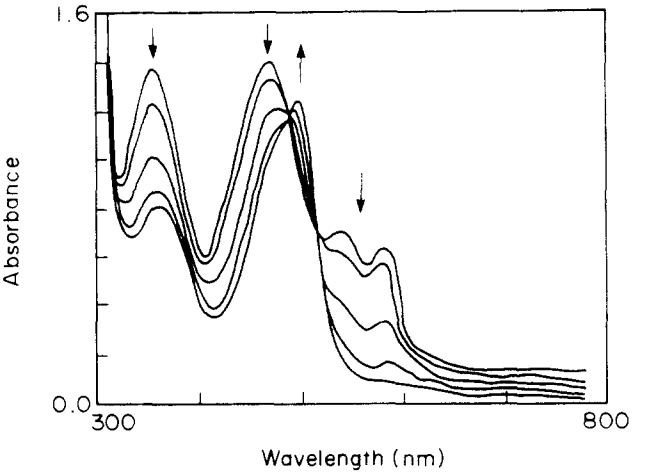

Fig. 5. Photoinduced reduction of HA: absorption spectra. Deaerated sample containing HA and BNAH in DMSO–buffer (1:1 by volume, pH 5.8) was illuminated for 0, 40, 80, 120 and 160 s. Arrows indicate the direction of changes.

The latter species could be formed in two one-electron transfer reactions or during disproportionation of $HA^{\bullet -}$ (Eq. (3)). Transfer of the second electron as well as the disproportionation process may be catalysed by protonation of the radical anion $HA^{\bullet -}$ at low pH [17]. Such an interpretation is consistent with the results of the EPR experiments in DMSO–buffer (pH 5–6), where no radical was detected (see above). The changes in concentrations of HA and BNAH before and after illumination were known by measuring the changes in absorbances at 540 nm (for HA, $\epsilon_{540\ nm} = 7.39 \times 10^3\ M^{-1}\ cm^{-1}$) and 356 nm (for BNAH, $\epsilon_{356\ nm} = 6900\ M^{-1}\ cm^{-1}$). Approximately 1 mol BNAH was consumed in photoreduction of 1 mol HA, i.e. the ratio of HA and BNAH is about 1:1. BNAH has generally been found to act as a net hydride donor in photoreductions [21]. Thus, a reasonable stoichiometry is obtained:

$$1HA + 1BNAH \longrightarrow 1HAH^- + 1BNA^+ \quad (4)$$

This further confirms that the photoreduced product is the hydroquinone HAH^-, and the new band at 496 nm is characteristic of HAH^-. Another support for this comes from the chemical reduction of HA with excess $Na_2S_2O_4$, in which similar absorption spectra to those in Fig. 5 were obtained. The absorption maximum of HAH^- is pH dependent and red shifts with increase in pH. For example, the absorption maximum of HAH^- is located at 496 nm in DMSO–buffer (1:1 by volume, pH 5.8) but in DMSO–buffer (1:1 by volume, pH 8.0) it is at 520 nm. This is characteristic of phenolic compounds.

Similar photoinduced optical changes for HA were observed when ascorbic acid was used instead of BNAH as reductant. This further supports that, under the reaction conditions employed in Ref. [10], the suppression of the EPR signal of the HA radical is due to

the acceleration of disproportionation of $HA^{\cdot -}$ and/or the transfer of the second electron to generate HAH^- but not due to the reaction of ascorbic acid with $HA^{\cdot +}$.

Photoinduced reduction in weak alkaline solution and dimethylsulphoxide

When a deoxygenated DMSO–buffer (1:1 by volume, pH 8.0) solution containing HA and BNAH was illuminated, the colour of the sample changed from red to green and then to pink–orange. Fig. 6(a) shows the absorption curves recorded during the colour change from red to green. It can be seen that the heights of the absorption of HA and BNAH decrease and a new band of the green intermediate at 628 nm appears, accompanied by one isosbestic point at 572 nm. On further illumination of the green intermediate, another new band at 520 nm is formed with a decrease in the height of the absorption of the green intermediate and two isosbestic points appear at 383 nm and 554 nm (Fig. 6(b)). The shape of the final absorption spectrum in Fig. 6(b) is very similar to that in Fig. 5, so it can be assigned to the photoproduct HAH^-. The shift in the absorption maximum of HAH^- from 496 nm (pH 5.8) to 520 nm (pH 8.0) is due to the effect of pH.

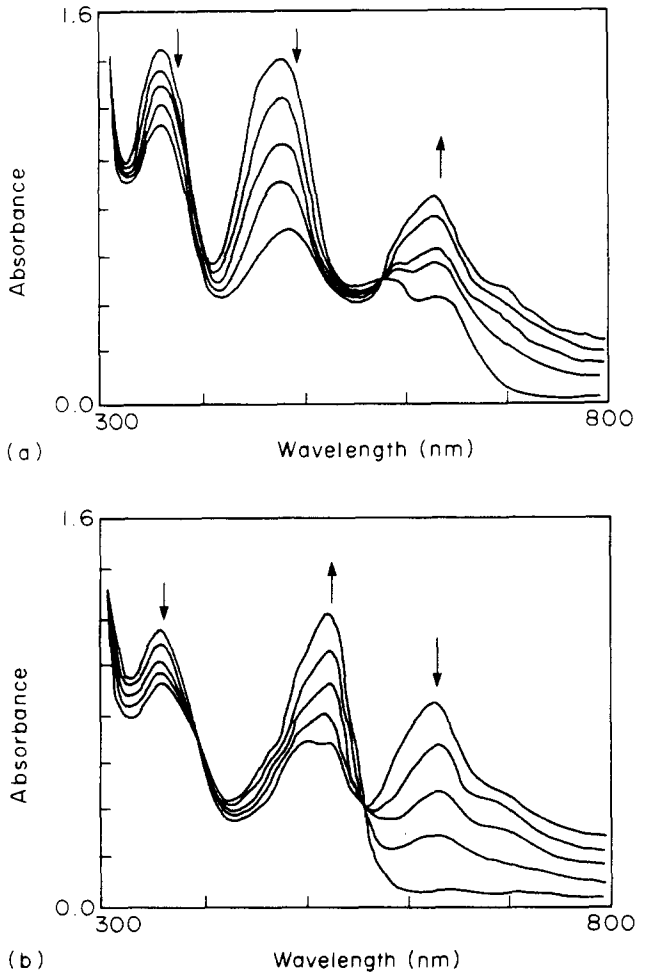

Fig. 6. Photoinduced reduction of HA: absorption spectra. Deaerated sample containing HA and BNAH in DMSO–buffer (1:1 by volume, pH 8.0) was illuminated for (a) 0, 5, 10, 15, 20 s and (b) 30, 45, 60, 75, 90 s. Arrows indicate the direction of changes.

When the same sample was illuminated in the EPR cavity, the EPR signal of $HA^{\cdot -}$ was detected immediately after illumination. It was also observed that, as the absorption at 628 nm increased, the intensity of the EPR signal of $HA^{\cdot -}$ increased concomitantly and the colour of the solution changed gradually from red to green. On further illumination the colour of the solution changed to pink–orange and the intensity of the EPR signal decreased. These observations confirm that the green intermediate species formed as shown in Fig. 6(a) is $HA^{\cdot -}$ and the new absorption band at 628 nm in Fig. 6(a) is characteristic of $HA^{\cdot -}$. To support this suggestion further a sample of HA and ascorbic acid was illuminated until a clear maximum at 628 nm developed, and a series of consecutive scans in the dark was performed in order to determine the rate of its decay. It was found that the slow decrease in the absorption at 628 nm is concomitant with the formation of the peak at 520 nm, characteristic of HAH^- (see above) probably via reaction (3). The decay trace measured by absorption spectroscopy follows second-order kinetics (Fig. 4(b)) and is parallel to that measured from the decay of the intensity of the EPR signal of $HA^{\cdot -}$ (Fig. 4(a)). This further confirms the correct assignment of the green intermediate with absorption maximum at 628 nm to $HA^{\cdot -}$.

Samples illuminated in DMSO, DMSO–buffer (1:1 by volume, pH 6.7) and DMSO–buffer (1:1 by volume, pH 7.3) mixtures produced spectra similar to those described above for pH 8.0 (Fig. 6). In all cases the absorption spectra of $HA^{\cdot -}$ were similar to one another and located at around 628 nm, indicating that the chromophores of the radicals at these pH values were in the same protonation state. This may be due to the existence of strong intramolecular bonds in $HA^{\cdot -}$. HA is capable of rapid tautomerization with the possibility of four different tautomers existing in solution at room temperature (Fig. 1). Similarly, $HA^{\cdot -}$ should also exist

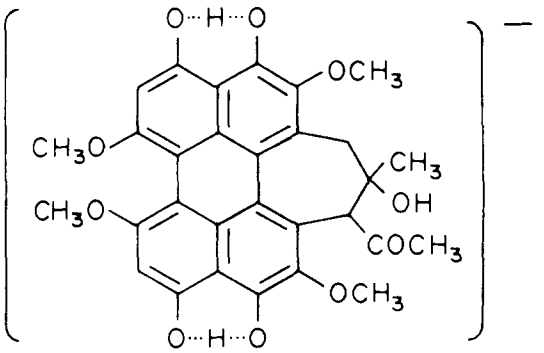

Fig. 7. Structure of the semiquinone radical anion of HA.

as four tautomers in solution. However, since these tautomers are in rapid equilibrium at room temperature [3,4], an average form was proposed to represent the structure of $HA^{\cdot -}$ (Fig. 7).

4. Conclusion

Illumination of HA in DMSO or DMSO–buffer (pH > 6.7) generated a strong EPR signal. This EPR signal was intensified in the presence of reductants, such as BNAH, NADH, reduced form of glutathione and ascorbic acid. The EPR spectrum was considered to originate from the semiquinone radical anion but not from the radical cation of HA [10]. In general, the radical cation of quinone is difficult to detect in common organic solvents and water owing to its strong oxidation ability. The detection of radical cation of quinone should be performed in solvents with high ionization potential, such as $CFCl_3$ [22], Freon-113 [23] and trifluoroacetic acid [24,25] at low temperature.

In contrast to the result obtained in neutral or weak alkaline solutions, no EPR signal of $HA^{\cdot -}$ was detected in the acidic mixture. This may be explained by a rapid transfer of a second electron to $HA^{\cdot -}$ and/or fast disproportionation of radicals, according to Eq. (3), facilitated by protonation of the radical anions. In agreement with this conclusion seems to be the observation that no transient species were detected in DMSO–buffer (pH 5.8) mixture using spectrophotometric methods.

The spectrophotometric measurements indicate that on illumination HA is directly reduced to its two-electron reduction product, i.e. hydroquinone (HAH^-), in acidic solution. The absorption maximum of HAH^- which is located at 496 nm at pH 5.8 shifts bathochromically with increase in pH value of the solution. However, in DMSO, neutral and alkaline DMSO–buffer (1:1 by volume) solutions it proceeds with one-electron reduction to generate its semiquinone radical anion ($HA^{\cdot -}$). The absorption maximum of $HA^{\cdot -}$ is at 628 nm in DMSO, neutral and alkaline DMSO–buffer solutions. On further illumination $HA^{\cdot -}$ is then reduced to HAH^-. Strong intramolecular hydrogen bonds are considered to exist in the chromophore of $HA^{\cdot -}$.

In summary, the identification of the semiquinone radical anion and hydroquinone of HA by EPR and absorption spectroscopies will facilitate future investigation on the properties of $HA^{\cdot -}$ and HAH^- and is important for understanding the biological functions of perylenoquinone derivatives. Studies on the detection of radical cation of HA in solvents with high ionization potential and on the reactions of $HA^{\cdot -}$ in biological systems are now in progress and the results will be reported in due course.

Acknowledgement

This research was supported by the National Natural Science Foundation of China.

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