*Biochimica et Biophysica Acta*, 637 (1981) 291–302
Elsevier/North-Holland Biomedical Press



BBA 48112

# IMMOBILIZED RADICALS

## IV. BIOLOGICAL SEMIQUINONE ANIONS AND NEUTRAL SEMIQUINONES

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(Received October 8th, 1980)
(Revised manuscript received April 7th, 1981)

*Key words: ESR; Semiquinone; Quinone; Immobilized radical*

**The semiquinone anion and neutral semiquinone radicals of benzoquinone, vitamin K-1, ubiquinone and plastoquinone were generated in both protic and aprotic solvents and frozen to produce immobilized spectra. The linewidths of the neutral semiquinones were always much larger than those of the corresponding anion radicals. Furthermore, the spectra of the neutral radicals often exhibit fine structure. When compared with in vivo spectra of semiquinones, these model systems suggest that the ubisemiquinone anion radical observed in photosynthetic bacteria can exist in either a protic or aprotic environment. There is also the implication that Signal II in chloroplasts may be a plastosemiquinone radical with a spin distribution similar to that of the neutral radical.**

## Introduction

The influence of environment on the ESR spectrum of a free radical in solution is a well known and highly characterized phenomenon. Hydrophobic and hydrophilic interactions, hydrogen bonding and ion pairing are just a few of the interactions that can perturb the ESR spectrum of a radical by broadening shifting or expanding it.

Observing and understanding environmental interactions in the powder spectra of immobilized radicals, however, are much more difficult. Although immobilization often favors these contact interactions, their presence in a spectrum frequently is obscured by the typical broadening encountered with powder samples. This broadening, of course, is not observed in solutions of low viscosity where the rapid tumpling of the radical averages the anisotropic components. In spite of these complications, we have shown [1–3] that a systematic study of immobilized radicals can yield environmental information from their powder spectra. For example, the spectrum of the immobilized benzosemiquinone anion [2] clearly shows the presence of two-site hydrogen bonding in protic solvents. This hydrogen bonding also produces an anisotropic dependency of the relaxation of the anion radical. This relaxation phenomenon [3] is most easily observed in deuterated systems at Q-band frequencies where the hyperfine interactions are suppressed and Zeeman interactions enhanced.

The knowledge gained from these model systems can now be used to investigate the environment of biological semiquinones in vivo. We have undertaken such a study on benzoquinone as well as three important biological quinones, vitamin K-1, ubiquinone-10 and plastoquinone-9.

The neutral semiquinones and semiquinone anions of these quinones were generated in protic and aprotic solvents, the solutions frozen and the ESR spectra recorded. Except for benzoquinone, the anions of the other three quinones show no fine structure. On the other hand, fine structure is observable in the spectra of many of the neutral radicals, the spectra of which are nearly twice as broad as their corresponding anions.

## Methods

*Rhodospirillum rubrum* (strain S-1) and *Rhodopseudomonas sphaeroides* (R-26 mutant) were grown photoheterotrophically using conventional methods [4,5]. AUT-e particles (i.e., iron-depleted phototrap complexes) were isolated from *R. rubrum* using the procedures of Loach et al. [6,7] while iron-depleted reaction centers were isolated from *Rps. sphaeroides* according to the method of Feher et al. [8].

*p*-Benzoquinone was obtained from Allied and purified by double vacuum sublimation. Ubiquinone-10 (Q-10) and vitamin K-1 were obtained from Sigma and used without further purification. Plastoquinone-9 was a gift from F. Hoffman-La Roche and Co. and was used as received. The structures of these quinones are shown in Fig. 1. All solvents were of spectroscopic grade purity and used without further purification. Deuterated ethanol was purchased from Stohler Isotope Chem.

Neutral semiquinone radicals were generated by irradiation with a 250 W Hg/Xe lamp degassed quinone solutions (concentration approx. $10^{-2}$ M) for 1 min in 2.5 mm outer diameter suprasil quartz ESR tubes. Directly after irradiation, the tubes were dropped into liquid $N_2$ to trap the radical in the frozen solvent matrix. These radicals were generated in either the aprotic solvent cyclohexane or the protic solvent ethanol. In order to ensure that only the neutral semiquinone was produced in ethanol, approx. $10^{-3}$ M acetic acid was added prior to irradiation.

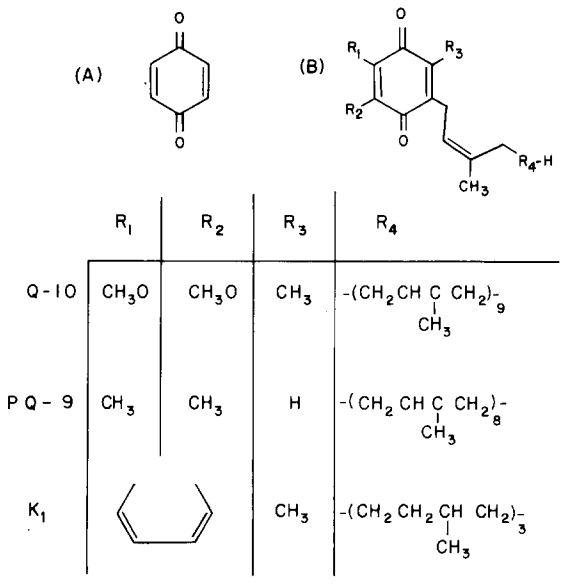



Fig. 1. Structures of *p*-benzoquinone (A) and three biological quinones (B): ubiquinone-10 (Q-10), plastoquinone-9 (PQ-9) and vitamin K-1 ($K_1$).

The semiquinone anion radicals were generated by chemical reduction of the parent quinone in ethanol containing approx. $10^{-4}$ M NaOH. The base concentration was always low enough to ensure that the *o*-quinone methide anion was not also generated (see Results). Reduction was allowed to occur for 1 min period prior to freezing in liquid $N_2$. We have verified that these techniques generate the neutral semiquinone and the semiquinone anion radicals by observing these radicals at room temperature in situ in the ESR spectrometer.

Spectra were recorded on a Varian E-109 ES ESR spectrometer resonating in a $TM_{110}$ mode. Samples were placed in a liquid $N_2$ insertion dewar flask (Varian) where boil-off bubbling was suppressed by blowing a slow strean of He gas through the liquid $N_2$. Microwave frequencies were determined to an accuracy of ±50 kHz with a JEOL cylindrical wavemeter positioned in the reflection arm of the spectrometer. Magnetic field values were determined to an accuracy of $\pm 1 \cdot 10^{-5}$ T with an NMR gaussmeter (Walker/Magnemetrics, Model G-502) using a Hewlett-Packard Model 5831A frequency counter to measure the resonance frequency of the probe. Microwave power used was 1 mW.

## Results

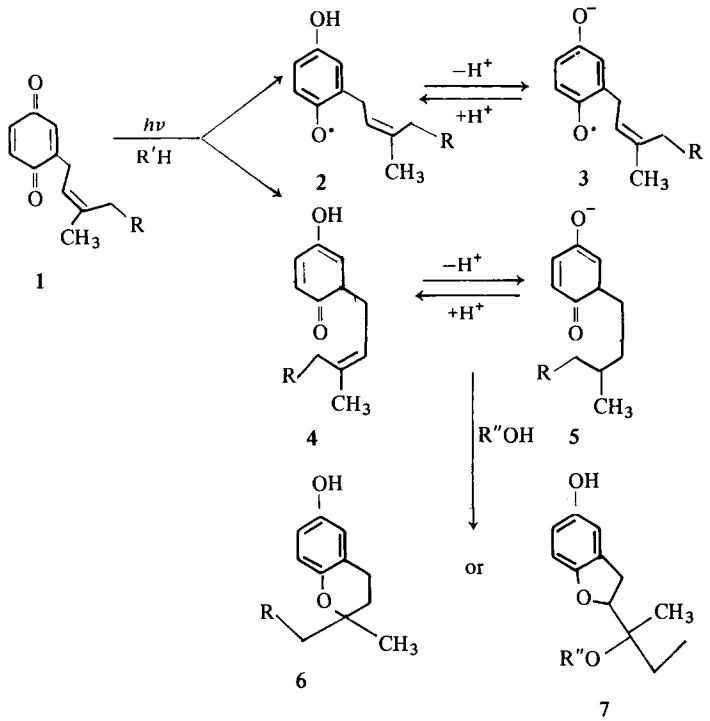
In order to understand fully the relationship between a quinone and its one-electron reduced semiquinone forms, it is best to understand first the different possible reactions of quinones, both biological and nonbiological. Since this subject is vast, only a summary of the photochemical reactions of quinones will be presented. An understanding of these reactions is especially important in this paper, since most semiquinone radicals are generated photochemically.

The basic photochemistry of *p*-benzoquinone is extremely simple in both protic and aprotic solvents. The initial reaction step is hydrogen abstraction from the solvent [9,10] by the photoexcited quinone in its triplet state producing the neutral semiquinone radi-

cal *. This radical decays by disproportionation to the quinone and hydroquinone (quinol). In basic protic media, the neutral semiquinone radical is deprotonated to form the longer lived semiquinone anion radical. Decay of the anion is presumably also by disproportionation.

The photochemistry of methylated quinones is slightly augmented from their unsubstituted parent [11,12]. Increasing methyl substitution and/or addition of water increases the $(\pi,\pi^*)$ character of the triplet and decreases the $(n,\pi^*)$ character. This change in the character of the reactive triplet introduces a second photochemical mechanism for its decay. For the methylated quinones, this mechanism is most observable with duroquinone (tetramethylbenzoquinone) and is the dominant reaction of the biological quinones. When photoexcited, a typical biological quinone (1) (Scheme I) will undergo one of two possible decay processes [13,14]. The first reaction has already been mentioned for benzoquinone, namely hydrogen abstraction from the solvent (RH) to form the neutral semiquinone radical (2) which is deprotonated in basic media to form the anion radical (3). The $pK_a$ for the equilibrium between 2 and 3 is generally 8–10 [9,10].

The second reaction of the photoexcited biological quinone is an intramolecular hydrogen abstraction by the excited triplet to produce the *o*-quinone methide (4) which can also deprotonate to produce the anion (5) with a $pK_a$ of 9–10 [13,14]. The *o*-quinone methide undergoes a slow decay generating either the chromenol (6) or the dihydrobenzofuran (7) [15].




Scheme I.

* There exists in the literature a nonuniformity in the naming of the one-electron reduced radicals of quinones. The term semiquinone radical has been used ambiguously to refer to either the anion or neutral radicals. To avoid this confusion, we will use the terms neutral semiquinone for the protonated semiquinone (2) and semiquinone anion (3).

*Semiquinone anions*

*Benzoquinone.* The spectrum of the immobilized benzoquinone anion ($BQ^-$) has already been thoroughly discussed by us [1–3]. A typical spectrum of $BQ^-$ frozen in ethanol is shown in Fig. 2. This is the

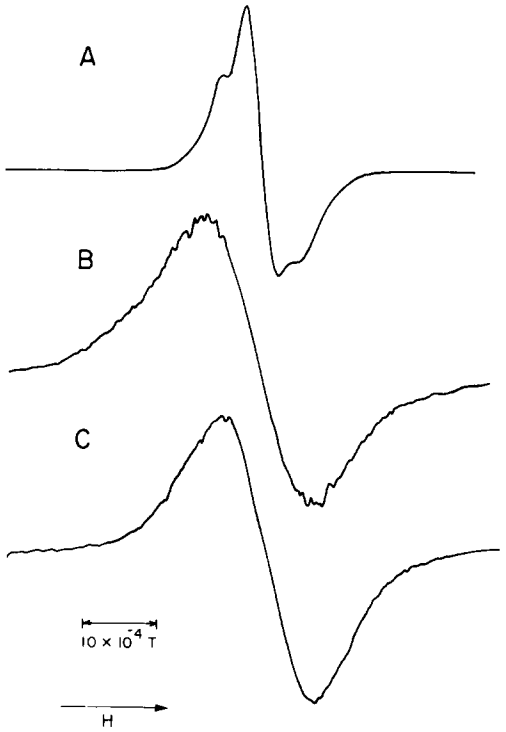



Fig. 2. First-derivative spectra of the immobilized semiquinone radicals of benzoquinone: (A) anion in ethanol, (B) neutral radical in ethanol, (C) neutral radical in cyclohexane. Modulation amplitude for this and subsequent figures is $0.8 \cdot 10^{-4}$ T.

TABLE I

ESR PARAMETERS OF *p*-BENZOSEMIQUINONE ANION SPECTRUM

| | $H_2O$ | Dimethyl sulfoxide | Ref. |
|---|---|---|---|
| $a^H$ | −2.357 G | −2.417 G | 38 |
| $a^{13}C$ (C-O) | +0.24 G | −2.13 G | 38 |
| $a^{17}O$ | −8.70 G | −9.46 G | 38 |
| $g_{iso}$ | 2.004659 | 2.005182 | 39 |

only semiquinone anion studied by us of which the immobilized spectrum shows fine structure. The isotropic spectral parameters of $BQ^-$ are highly solvent dependent as can be seen in Table I. Unfortunately, we are unable to observe the isolated semiquinone anion in nonpolar solvents. The anion can be generated in these solvents, but its spectrum is always perturbed due to its existence as an ion pair with its counter cation [3]. Because of this, all of the anions discussed have been generated in polar solvents. The principal spectral characteristics of the immobilized benzoquinone anion are listed in Table II along with the parameters of remaining anions.

*Vitamin K-1.* The generation of anion radicals of the various biological quinones is not straightforward.

TABLE II

SPECTRAL PARAMETERS OF SEMIQUINONE ANIONS

$A_L/A_H$, absolute magnitude of ratio of amplitude of low-field maximum to high-field minimum; BQ, benzoquinone; PQ, plastoquinone; RC, reaction center protein.

| Quinone (solvent) | *g* factor | $\Delta H_{pp}$ ($10^{-4}$ T) | $A_L/A_H$ | Ref. |
|---|---|---|---|---|
| BQ (ethanol) | 2.0047 ± 0.0002 | 5.4 ± 0.2 [a] | 1.19 | this work |
| Vitamin K-1 (ethanol) | 2.0046 ± 0.0002 | 8.5 ± 0.2 | 0.86 | this work |
| Q-10 (ethanol) | 2.0046 ± 0.0002 | 8.2 ± 0.2 | 1.01 | this work |
| Q-10 ([$^2H_6$]ethanol) | 2.0050 ± 0.0002 | 7.4 ± 0.2 | 1.14 | this work |
| Q-10 (AUT-e, *R. rubrum*) | 2.0050 ± 0.0003 | 7.0 ± 0.3 | 1.09 | 7, this work |
| Q-10 (RC, R-26, *Rps. sphaeroides*) | 2.0046 ± 0.0002 | 8.1 ± 0.4 | 1.01 | 8, this work |
| Q-10 (AUT-s R-26, *Rps. sphaeroides*) | 2.0048 ± 0.0002 | 7.9 ± 0.4 | 1.09 | 22 |
| Q-*n* (chromatophores, *R. rubrum*, pH 9.5) | 2.0046 ± 0.0002 | 8.1 ± 0.2 | 1.01 | this work |
| PQ-9 (ethanol) | 2.0046 ± 0.0002 | 9.5 ± 0.3 | 0.92 | 17, this work |

[a] Width from maximum amplitude in spectrum to minimum.

Unlike benzoquinone, the anion of which can be generated chemically, photochemically or electrochemically, biological quinones can undergo various side reactions as outlined at the beginning of this section.

We found that photochemical generation of the anion radical (3) always produced complex ESR spectra. First of all, the quantum yield [13,14] for the formation of the neutral semiquinone (2) which is the precursor to the semiquinone anion (3) is much less than that for the quinone methide (4). Also, the production of 4 is unaffected by the presence of $O_2$ while 2 is quenched. Since 4 has a strong absorption in the blue region of the visible spectrum, generation of 4 will mask the absorption of the parent quinone (1). Finally, subsequent photochemical excitation of the products 6 and 7 which may occur during continuous irradiation produces the radical anions of these two species [16]. Because of these reasons, photochemical excitation was never used to generate the radical anions of the biological quinones.

The easiest technique for generating the radical anion (3) is chemical reduction with $NaBH_4$ of the parent quinone in a slightly basic medium. It should be remembered that the methide anion (5) can be produced chemically [13,14] in basic media. It typically has a strong blue hue (compared to red for 4 and yellow for 3). If the system is made too basic prior to reduction, all of the quinone will be transformed chemically into 5. Therefore, the pH must be low enough to prevent the chemical generation of 5 but great enough to stabilize 3. Only trial-and-error testing for each quinone can determine the correct base concentration.

The spectrum of the immobilized vitamin K-1 anion in ethanol is shown in Fig. 3. It can be characterized as a nearly symmetric gaussian line of width $8.5 \pm 0.2 \cdot 10^{-4}$ T showing no observable fine structure (Fig. 4a). This line shape and width are the same for the vitamin K-1 radical anion generated in methanol and *n*-propanol.

*Ubiquinone.* The ESR spectrum of the semiquinone anion of Q in ethanol is shown in Fig. 5. Like

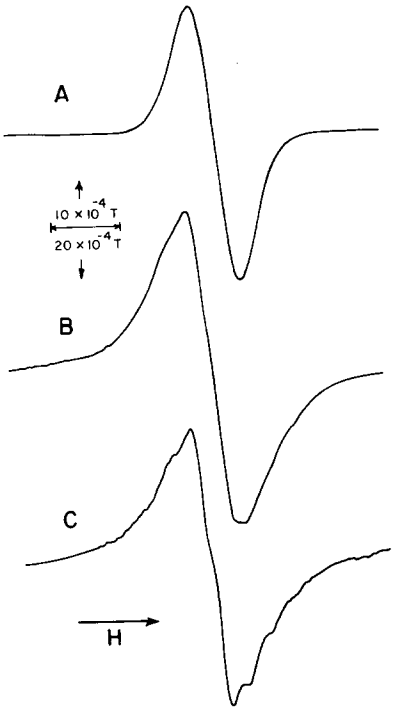



Fig. 3. First-derivative spectra of the immobilized semiquinone radicals of vitamin K-1: (A) anion in ethanol, (B) neutral radical in ethanol, (C) neutral radical in cyclohexane.

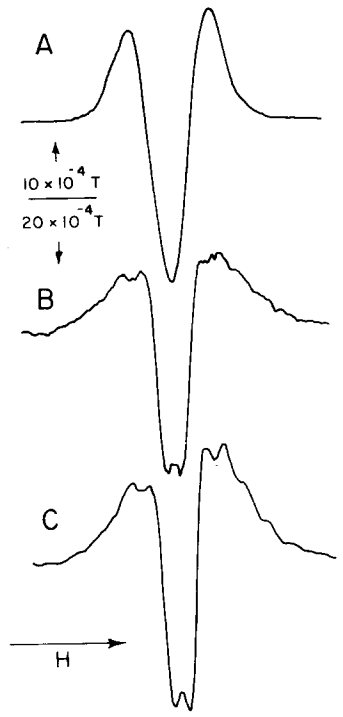



Fig. 4. Second-derivative spectra of the immobilized semiquinone radicals of vitamin K-1 shown in Fig. 3: (A) anion in ethanol, (B) neutral radical in ethanol, (C) neutral radical in cyclohexane. Note fine structure in B and C.

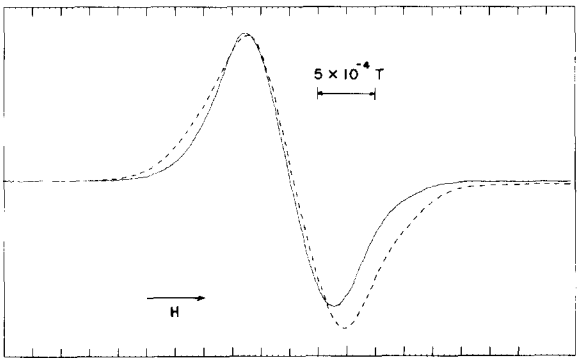



Fig. 5. First-derivative spectra of immobilized ubisemiquinone anion in ethanol (------) and [$^2H_6$]ethanol (———) at 77 K.

the anion radical of vitamin K-1, the spectrum of the immobilized anion is a symmetric gaussian line of width $8.2 \pm 0.2 \cdot 10^{-4}$ T. Since the anion radical of Q has been observed in a large number of biological systems, it is of interest to determine the influence of protic environments on the spectrum of this immobilized radical. It has been shown previously by us [1,2] that the spectrum of the immobilized anion of benzoquinone is greatly narrowed when generated in deuterated protic media. The explanation for this phenomenon is that solvent hydrogen bonding broadens the immobilized radical's spectrum by incorporating additional hyperfine structure from the solvent's hydrogen bond. Therefore, the smaller magnetic moment of a deuteron in a deuterated solvent will have less of a broadening effect on the anion's spectrum. Because of this, deuterated solvents can be used to mimic the spectrum of the anion in an aprotic environment.

Fig. 5 also shows the spectrum of the immobilized anion radical of Q in perdeuterated ethanol. There are three important spectral changes that occur in deuterated solvents; all of them are related. First of all, the spectrum narrows. This is understandable since, as stated above, deuteration of the solvent suppresses the fine structures of the hydrogen bond. Secondly, there is a shift of the $g$ factor of the crossover point of the spectrum. Narrowing the spectrum through deuteration of the solvent accentuates the $g$ factor anisotropy of the radical. As this narrowing increases, the crossover of the spectrum shifts from the average or isotropic $g$ factor to the intermediate principal $g$ factor. For quinones the intermediate $g$ factor is greater than the isotropic value [1]. Therefore, narrowing will cause the spectrum to shift to a larger $g$ factor as the effects of hydrogen bonding are suppressed.

Finally, deuteration of the solvent causes the spectrum of the immobilized anion radical of Q to become asymmetric. Since the $g$ factor anisotropy becomes more observable with solvent deuteration and since the intermediate $g$ factor is larger than the isotropic value, the spectrum will not narrow symmetrically. In other words, the $g$ factor anisotropy is more obvious on one side of the spectrum than the other thus producing an asymmetric narrowing. It should again be emphasized that all of these effects are related to the same cause. Their presence in the spectrum of an immobilized anion radical can be interpreted as implying that the radical is in an aprotic environment.

*Plastoquinone.* The spectrum of the immobilized radical anion of plastoquinone has already been

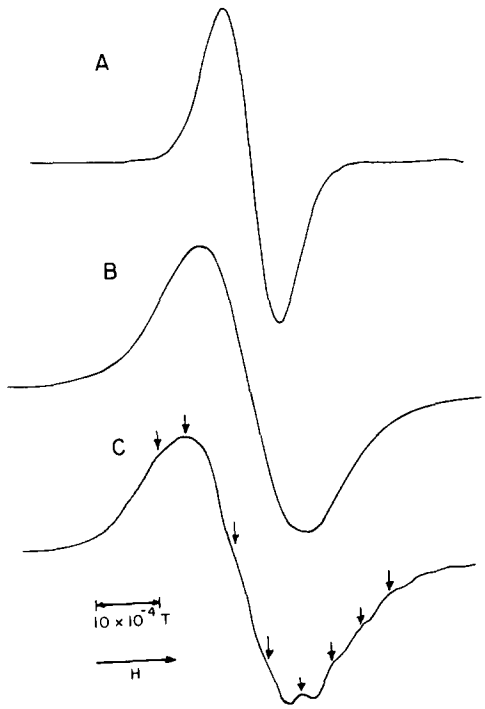



Fig. 6. First-derivative spectra of the immobilized semiquinone radicals of plastoquinone-9: (A) anion in ethanol, (B) neutral radical in ethanol, (C) neutral radical in cyclohexane.

investigated by Kohl et al. [17]. Although our technique for generating the anion is different from theirs, the spectrum obtained is essentially the same. It consists of a single-lined symmetric gaussian signal of width $9.5 \pm 0.3 \cdot 10^{-4}$ T (see Fig. 6 and Table II).

It was mentioned above that the linewidths of the spectra of immobilized semiquinone anions in protic solvents are broadened through hydrogen bonding with the solvent. We have also shown [3] that this hydrogen bonding produces an anisotropic saturation which arises from the directional dependency of the hydrogen bonding in the system. Typically, the hydrogen bonding changes the saturation behavior of two of the three principal directions. For the benzosemiquinone anion, this effect is observable [3] as a more rapid saturation of the center of the spectrum relative to the wings.

It is of interest therefore, to see whether or not this phenomenon is also observable in the immobilized spectra of the biological semiquinone anions. Fig. 7 shows the effect of power on the spectrum of the anion of plastoquinone at 10 K. The asymmetric broadening and shift of the center of the spectrum again imply that an anisotropic saturation mechanism is operating in this system. Therefore, hydrogen bonding broadens the spectra of biological semiquinone anions in protic environments and induces an anisotropic saturation. These effects are not observed in the spectra of the immobilized neutral semiquinones discussed below.

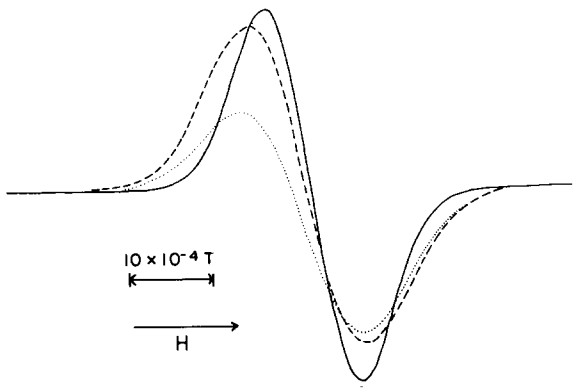



Fig. 7. Effect of power on the spectrum of the immobilized plastosemiquinone anion radical in ethanol at 10 K. Microwave power: 0.2 mW (———), 10 mW (- - - - -), 20 mW (· · · · ·).

*In vivo anions.* There are many examples in the literature of ESR spectra assigned to semiquinone anion radicals observed in vivo in both photosynthetic bacteria and mitochondrial electron-transport complexes. Unfortunately, many of these spectra were not characterized by the authors in terms of linewidth and $g$ factor to the number of significant figures used in this work and, therefore, cannot be accurately compared.

There are, however, some semiquinone anion spectra in biological systems which have been well characterized. One example is the primary electron acceptor in photosynthetic bacteria. It is generally felt that the primary electron acceptor is an iron-quinone complex (typically ubiquinone) in the reaction center protein. Furthermore, it has been shown recently [18] that the secondary acceptor is also a quinone probably complexed to the same iron but having a slightly higher redox midpoint potential than the primary quinone. When reduced, this primary complex yields an ESR spectrum [19] similar to that of ferredoxins. On the other hand, removal of iron from reaction centers allows the observation [7,8] of the unperturbed reduced primary quinone. It is in this iron-free quinone spectrum that we are interested.

There are two main procedures for producing iron-free reaction centers. The first was devised by Loach et al. [6,7] and uses electrophoretic separation with ampholites to chelate the primary iron which has been loosened by a detergent-base treatment and yields a particle referred to as AUT-e. The second procedure, devised by Feher et al. [8], involves the use of two detergents (lauryldimethylamine-*N*-oxide and sodium dodecyl sulfate (SDS)) to loosen the reaction center protein and release the iron. There is also a third procedure devised by Slooten [20,21] which is similar to the two above-mentioned techniques. In this procedure, reaction centers are first solubilized in 0.3% SDS as in the procedure of Feher et al. [8], then centrifuged as in the AUT technique of Loach et al. [6,7]. The resultant particles are called AUT-s. The ESR spectral properties of this signal have been recently investigated by Gast and Hoff [22]. All these preparations yield a system which exhibits a light-induced semiquinone signal at room temperature. An example of the Q anion in the AUT-e preparation is shown in Fig. 8.

We have reproduced these preparations and care-

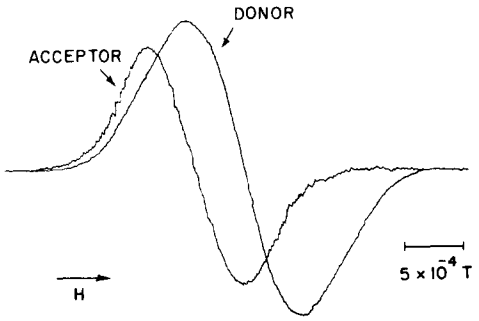



Fig. 8. Spectrum of ubisemiquinone anion (acceptor) in reduced iron-depleted phototrap complexes (i.e., AUT-e particles) from *R. rubrum*. Also shown in figure is the spectrum of oxidized bacteriochlorophyll donor from the same particles. Spectra were taken at room temperature.

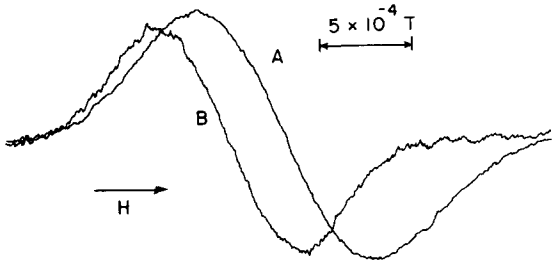



Fig. 9. Spectrum of probably ubisemiquinone anion signal (B) obtained by reduction of chromatophores of *R. rubrum* at pH 7.5 at room temperature. For comparison, the spectrum of the donor bacteriochlorophyll cation (A) obtained from the same preparation is also shown.

fully measured the spectroscopic parameters of each. These are listed in Table II. As can be seen, the linewidth, $g$ factor and asymmetry of these signals are different. All of these differences can be understood easily in terms of the semiquinone system where hydrogen bonding contributes significantly to the overall spectral linewidth. As discussed above, this model shows that as the hydrogen bonding interaction increases, the spectral linewidth increases masking the anisotropy. This broadening produces a more symmetric line with a $g$ factor closer to that of the isotropic value.

When the spectral parameters from the solution Q semiquinone anions are compared with those of the in vivo signal (see Table II), it can be inferred that the semiquinone signal observed in the AUT-e preparation is in a more aprotic environment than those of the reaction center protein preparation while the environment of the AUT-s seems to be between these two. Analysis of the quinone concentration in the first two of these preparations [23,24] by chromatographic analysis of solvent extracts suggests that there is approx. one Q per reaction center in the reaction center protein preparation while there are at least two in the AUT-e preparation. On the other hand, the semiquinone signal in either preparation is unaffected by the presence of *o*-phenantholine. At this time, it is difficult to say which, if any, iron-free preparation reflects the environment of the true iron-complexed primary quinone acceptor.

The primary quinone acceptor is not the only quinone in photosynthetic bacteria. It is strongly felt that the majority of ubiquinones in photosynthetic bacteria exist in a membrane- or protein-bound pool [25]. As mentioned above, a semiquinone radical in this pool has probably not been observed because of its rapid decay by disproportionation. This would be similar to the reaction observed in semiquinone solutions where the rate of decay is diffusion limited for the neutral radical and 2 orders of magnitude slower for the anion. Several years ago we showed [26] that a stable semiquinone could be observed in chromatophores of *R. rubrum*. Since the radical anion is known to be stabilized in protic solvents of high pH, we suspended chromatophores in phosphate buffer at pH 9.5. We showed that either photolysis or chemical reduction generated a stable radical (Fig. 9) with spectral parameters (Table II) very close to those determined for the ubisemiquinone anion. Furthermore, Table II implies that this radical is a ubisemiquinone anion in a protic environment. It is now felt that this signal is due to a protein- or a membrane-bound quinone which is not the primary or secondary quinone acceptors of which the spectra are dramatically changed by the presence of a neighboring iron atom.

*Neutral semiquinones*

*Benzoquinone.* All of the neutral semiquinone radicals were generated photochemically. Except after extremely long irradiation periods (approx. 1/2 h), no detectable secondary radical generation was observed. The main problem with generating the neutral radical is its short (diffusion controlled) rate

TABLE III

SPECTRAL PARAMETERS OF NEUTRAL SEMIQUINONES

BQ, benzoquinone; PQ, plastoquinone; n.d., not determined.

| Quinone (solvent) | *g* factor | $\Delta H_{pp}$ ($10^{-4}$ T) | Line shape | Ref. |
|---|---|---|---|---|
| BQ (ethanol) | 2.0048 ± 0.0003 | 15.5 ± 0.4 | symmetric, gaussian | this work |
| BQ (cyclohexane) | 2.0048 ± 0.0003 | 12.5 ± 0.3 | symmetric, gaussian | this work |
| Vitamin K-1 (ethanol) | 2.0049 ± 0.0003 | 16.0 ± 0.3 | fine structure | this work |
| Vitamin K-1 (cyclohexane) | n.d. | 13.5 ± 0.3 | 8–9 lines, fine structure | this work |
| Q-10 (ethanol) | 2.0048 ± 0.0002 | 12.0 ± 0.3 | nearly symmetric, single line | this work |
| Q-10 (cyclohexane) | n.d. | 11.5 ± 0.3 | assymmetric, single line | this work |
| PQ-9 (ethanol) | 2.0046 ± 0.0002 | 18.0 ± 0.3 | symmetric, gaussian | this work |
| PQ-9 (cyclohexane) | 2.0048 ± 0.0002 | 17.5 ± 0.3 | 8–9 lines, fine structure | this work |
| Signal II (spinach) | 2.0046 ± 0.0002 | 19.3 ± 0.3 | 5 lines | 17, this work |
| Signal II (collard greens) | n.d. | 19.0 ± 0.3 | 5 lines | this work |
| Chromatophores, *Rps. sphaeroides*, pH 6.0 | n.d. | 13.5 ± 1.0 | n.d. | 25 |

of decay. Because of this, samples had to be rapidly frozen after irradiation in order to trap the neutral radical. Samples were never irradiated while frozen, since this trapped both the neutral radical and the solvent radical.

Fig. 2 shows the spectra of the neutral radical of benzoquinone in both ethanol and cyclohexane. In most cases, the ethanol was made slightly acidic to ensure production of only the neutral radical and not the anion. There are two important general features (see Table III) about these spectra. Firstly, the linewidths are much larger for the neutral radicals than they are for the anions. Secondly, the linewidth of the radical in the aprotic, nonpolar solvent cyclohexane is smaller than that in ethanol. These two effects were consistently observed for all the quinones investigated. Table III lists all of the spectral characteristics of the neutral semiquinones studied.

*Vitamin K-1.* Unlike the anion, the spectrum of the neutral semiquinone of vitamin K-1 in both ethanol and cyclohexane shows the presence of fine structure (Fig. 3 and 4). This structure is more obvious in the spectrum of vitamin K-1 in cyclohexane where nine to ten lines are observable with an average splitting of approx. $6 \cdot 10^{-4}$ T.

*Ubiquinone.* Despite being broader than the anion radical, the neutral semiquinone radical of Q is also highly asymmetric (Fig. 10). In fact, in cyclohexane there is a noticeable distortion of the spectrum. One possible cause of this extreme distortion is that the methoxy group contributes very little hyperfine interaction [27] to the spectrum of the ubisemiquinone

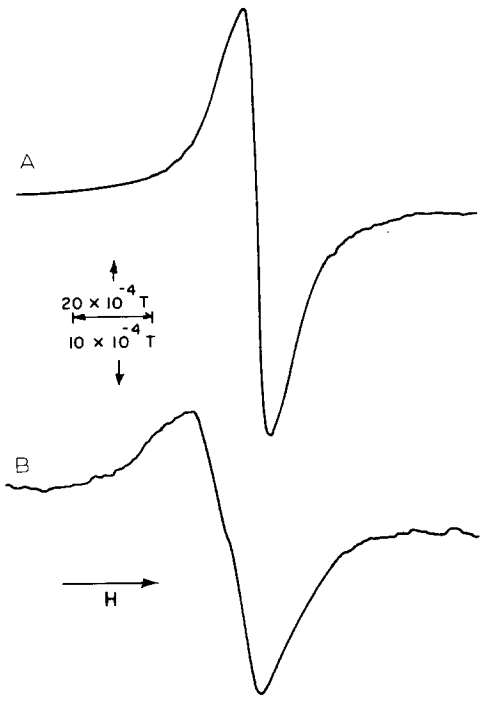



Fig. 10. First-derivative spectra of neutral semiquinone of ubiquinone in (A) ethanol and (B) cyclohexane.

radical. Because of this, the line shape of the powder spectrum is influenced more than usual by the $g$ factor anisotropy, thus leading to the overall observed distortion.

*Plastoquinone.* Fig. 6 shows the powder spectra of the neutral radical of plastoquinone in both ethanol and cyclohexane. This radical exhibits the largest linewidth of all the radicals observed by us. The broader spectrum in ethanol contains no detectable fine structure while the spectrum of the radical in cyclohexane clearly contains many inflection points. These inflections (approx. eight to nine; see arrows, Fig. 6c) are about $6 \cdot 10^{-4}$ T apart. The room temperature spectrum of this system (unpublished results) shows that it corresponds to the neutral radical where protonation has occurred *meta* to the isoprenoid side chain.

*In vivo neutral semiquinones.* To date, no quinone spectra detected in living systems have been attributed to neutral semiquinones. One reason for this void is that no one knows what a neutral semiquinone spectrum looks like. A possible candidate is Signal II from plant systems.

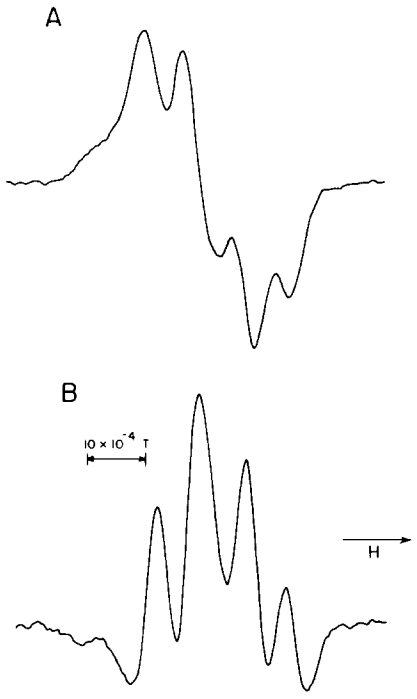



Fig. 11. First- (A) and second- (B) derivative room temperature spectra of Signal II from spinach chloroplasts. Microwave power, 0.5 mW; modulation amplitude, $0.5 \cdot 10^{-4}$ T.

Signal II was first observed in spinach chloroplasts in 1956 by Commoner et al. [28]. Fig. 11 shows first- and second-derivative spectra of Signal II from spinach. It is clear from this figure that this is a five-lined signal with possible secondary inflections on some of the lines. Signal II is light induced with several different decaying components [29–34]. Within the spectrometer's sensitivity limits, all of these components appear to have the same spectrum.

The identity of Signal II is not known, although generally it is felt to be associated with a plastoquinone radical or a radical derivative of plastoquinone. Probably the strongest support of this theory has come from the work of Kohl and co-workers [17,35]. They showed that solvent extraction of plastoquinone from chloroplasts eliminated Signal II while subsequent back-addition with deuterated plastoquinone yielded a narrowed Signal II. We have shown above that the powder spectrum of the chemically generated plastosemiquinone anion was much narrower than Signal II and lacked fine structure. Because of this, they proposed that Signal II is a plastochromenoxyl radical which has a powder spectrum similar to Signal II.

The chromenoxyl radical which they propose as Signal II typically is generated from the plastochromenol [12–14], a plastoquinone derivative which has not been isolated from green plants. As discussed earlier, the chromenol (6) so far has been shown to be produced only from plastoquinone by direct ultraviolet irradiation. It is not known if this reaction occurs in green plant systems in the absence of ultraviolet irradiation or if the deuterated plastoquinone in the experiments mentioned above could be quickly converted to the plastochromenoxyl radicals during reconstitution.

Another possible complication arises from the works of Ruuge et al. [36] and Nishi et al. [37] who showed that Signal II possesses complex saturation behavior and may be due to the spectral overlap of several different radical species. These papers are more fully discussed in the following article and are not immediately relevant here.

A point that is seldom raised when discussing semiquinone spectra in vivo is the possibility of seeing the protonated form (2). The $pK_a$ of the reaction between 2 and 3 has been determined by Takamiya and Dutton [25] to be 8.5 in vivo for ubiquinone.

This value is not know for other quinones but it is probably safe to assume that it is in the range 8–10 in most biological environments. Since physiological pH is considered to be lower than this, it is not inconceivable that the local pH of a region could be low enough to shift the equilibrium toward 2.

Obviously, the spectrum of the immobilized neutral radical of plastoquinone (Fig. 6) does not look like Signal II. The linewidths are similar, but Signal II shows more detailed structure than the neutral radical of plastoquinone. Even though the radicals are not the same, the closeness of the overall linewidths implies that Signal II has a spin distributions more similar to the plastoquinone neutral semiquinone than the plastoquinone anion radical. Specifically, the more uniform spin distribution of the radical anion yields a spectrum too narrow while the highly asymmetric distribution of the neutral radical gives a spectrum much closer to that of Signal II in the in vivo system.

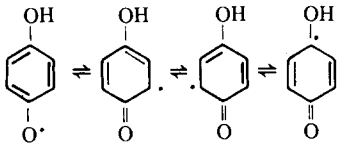
## Discussion

When we commenced our study of immobilized radicals, we stated that we wished to investigate the spectra of model in vitro systems in order to understand better the environment of radicals detected in vivo. Unfortunately, subtle differences are often underestimated or even disregarded in powder spectra of similar radicals observed in different biological systems. Hopefully, this paper will show that a radical's powder spectrum can contain important information concerning its environment.

For example, when compared with in vivo spectra, the spectra of the immobilized ubisemiquinone anion demonstrate the existence of at least two different sites for Q in the electron-transport system. The fact that the $Q^-$ spectrum is different in the AUT-e, AUT-s and reaction center protein preparations suggests that either these quinones are different (i.e., primary or secondary acceptors) or that the binding site of one or all has changed during iron extraction. If this is true, one should be warned that differences in any of the physical measurements of the primary reaction that have been measured in these various iron-depleted systems may be due to these different environments.

Our present understanding of the electron-transport system in photosynthetic bacteria implies that quinones exist as the primary, secondary and possibly tertiary electron acceptor of the photochemical reaction as well as in a large quinone pool. Since we have shown that several of these quinone species are in different (i.e., protic or aprotic) environments, their differences in their redox midpoint potential may be a result of this environmental difference.

This research also clearly demonstrates that none of the in vivo ubisemiquinone radicals that have been observed in the primary bacterial photosynthetic reaction are protonated. This is significant, since it is felt [25] that both the primary and secondary quinone acceptors can be protonated with $pK_a$ values of 9.8 and 8.5, respectively, in *Rps. sphaeroides*. Table III shows that protonation always increases the spectral linewidth. This increase is due to the asymmetric perturbation of the spin density on the quinone ring. By protonating one of the phenolate oxygens of the semiquinone, the spin density on the quinoid ring *meta* to protonation typically doubles due to the resonance:



It is presumably this increase which produces the large increase of the spectral linewidth of the immobilized radical. Since hydrogen bonding has been shown [1–3] to occur at both phenolate groups of the semiquinone anion in protic media, it does not produce an asymmetric perturbation of the anion's spin density as does protonation. It does, however, broaden the spectrum. This broadening, or lack of it, can be used to monitor the protic nature of the environment of the various biological semiquinone anions in vivo.

In general, the spectra of the neutral semiquinones show a greater variation in linewidth and fine structure than do the spectra of the anion radicals. This fact should allow for a more definitive identification of the ESR spectrum of neutral semiquinones in vivo. To date, however, no such spectra have been observed. As mentioned above, Signal II in green plant systems may be a neutral plastoquinone radical. Although not identical to the spectrum of the plastoquinone neutral semiquinone, Signal II has a linewidth much closer to that of this radical than that of the semiquinone

anion. The closeness of these spectra does imply the plastoquinone radical giving rise to Signal II has a spin density distribution much closer to that of the neutral radical than the anion.

Finally, Takamiya and Dutton [25] have recently analyzed the quinones of chromatophores of *Rps. sphaeroides*. Using ESR spectroscopy, they were able to detect a signal at pH 6.0 which could possibly contain the ubisemiquinone radical. The linewidth measured from their figure of the radical, assuming a microwave frequency of 9.1 GHz, is $13.5 \pm 1.0 \cdot 10^{-4}$ T. This value is very close to that of the Q neutral radical measured by us (Table III). If this signal they observed were the neutral Q radical, it could be the same quinone the anion of which we have observed (Fig. 9) at higher pH. It is suggested by them that this may be a loosely bound ubiquinone molecule not associated with the large membrane quinone pool but possibly with Q-cytochrome $b/c_2$ oxidoreductase.

## Acknowledgement

This research was supported by grant GM 25049 from the National Institute of General Medical Sciences of the Public Health Services.

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