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# The Involvement of Biological Quinones in the Formation of Hydroxyl Radicals via the Haber–Weiss Reaction

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The present investigation was made to evaluate biologically relevant quinones as possible catalysts in the generation of hydroxyl radicals from hydrogen peroxide and superoxide radicals. ESR spectra demonstrated that menadione, plastoquinone, and ubiquinone derivatives could all be reduced to their semiquinone forms by electron transfer from superoxide radicals. Reductive homolytic cleavage of $H_2O_2$ was observed to be dependent upon the presence of semiquinones in the reaction medium. Our data strongly support the concept that quinones normally involved in physiological processes may also play a role as catalysts of the Haber–Weiss reaction in the biological generation of hydroxyl radicals. 

## INTRODUCTION

Since the detection of biological sources of $O_2^{\cdot-}$ and $H_2O_2$, a great number of cytotoxic effects have been explained by the formation of $OH^{\cdot}$ radicals. Suggestions that these radicals are implicated in toxic actions of oxygen have been inferred from studies with selective radical scavengers. The involvement of $O_2^{\cdot-}$ and $H_2O_2$ in the formation of highly reactive oxygen species has been concluded from observations of partial inhibitory effects of a combination of both enzymes. This concept was further based on a chemical reaction known for many years as the "Haber–Weiss reaction":

$$O_2^{\cdot-} + H_2O_2 \rightarrow OH^{\cdot} + OH^{-} + O_2 \qquad [1]$$

However, controversy has arisen about the existence of such a pathway in biological systems, because the reaction was shown to be extremely slow at the low concentrations of $O_2^{\cdot-}$ and $H_2O_2$ likely to be present *in vivo* (*1, 2*). Walling proposed ferrous iron as a catalyst of $OH^{\cdot}$ formation from $H_2O_2$ (*3*). Furthermore, a permanent source of $O_2^{\cdot-}$ radicals is required in this reaction cycle to reduce ferric iron to its ferrous form. As an analogy to chemical and biochemical studies with the iron-catalyzed Haber–Weiss reaction, the involvement of coordinated iron was also suggested as a basis for biological formation of the $OH^{\cdot}$ radical (*4, 5*).

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We have recently shown that mitochondrial formation of $OH^{\cdot}$ radicals was not sensitive to potent iron chelators such as deferoxamine, DETAPAC (diethylenetriamine pentaacetic acid), and bathophenanthroline disulfonate, but appeared to depend on steady-state concentrations of ubisemiquinone radicals (*6, 7*). Further approaches to demonstrate the involvement of ubiquinone in the reductive cleavage of $H_2O_2$ were made by following $OH^{\cdot}$ formation from $H_2O_2$ in ubiquinone-extracted and ubiquinone-reconstituted rat heart mitochondria. The results of these experiments were unsatisfactory because of structural and functional disorganization of respiratory components by the extraction procedure for ubiquinone. Furthermore, ubiquinone (UQ), normally present in a 5- to 6-fold excess relative to the cytochromes could not be removed from the respiratory chain by more than 90%.[2] In order to avoid difficulties related to the complexity of the mitochondrial membrane system we designed defined conditions which permit the evaluation of the catalytic activity of coenzyme Q-derivatives in the formation of $OH^{\cdot}$ from $H_2O_2$ and $O_2^{\cdot-}$. In addition to ubiquinones other physiological quinones were also investigated for their capacity to shuttle electrons from $O_2^{\cdot-}$ to $H_2O_2$.

## MATERIALS AND METHODS

All quinones under study were gifts. Ubiquinone derivatives (UQ-n) came from Hoffman–LaRoche (Switzerland), UQ-0 was from Dipl. Chem. T. Link (Munich), and plastoquinone (PQ-1) was from Professor Hauska (Regensburg, West Germany). Menadione and DETAPAC were purchased from Sigma Chemical Co. (Munich). All ubiquinone derivatives were purified by means of low-pressure chromatography using a reversed-phase column (LiChroprep RP-8-ic from Merck, Darmstadt, West Germany), and ethanol (98%) for the elution. Purified UQ fractions, identified by an UV detector at 275 nm, were separated by thin-layer chromatography (RP-18-HPTLC, Merck) and dissolved in acetonitrile. DMPO (5,5-dimethylpyrroline-*N*-oxide) from Aldrich-Europe (Belgium) was purified and stored as follows. Purification of the DMPO spin trap was performed with 10 g activated charcoal (GR/10 ml) and 0.15 mol/liter KCl, 50 mmol/liter Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.4). Prior to the addition of 1 g DMPO the medium was cooled to 0°C, and saturated with oxygen-free nitrogen. After 10 min continuous bubbling with nitrogen in the dark, the spin trap was recovered by filtration and stored at −20°C in KCl–Hepes buffer equilibrated with nitrogen. Acetonitrile was purified with a molecular sieve (3 Å, Merck) to remove contaminating water. Crown ether (2,5,8,15,18,21-hexaoxatricyclo-(20,4,0,0,9,14)-hexacosane) came from Merck, and potassium superoxide from Fluka AG (Buchs, Switzerland).

All ESR measurements were performed under nitrogen (unless otherwise indicated) with a Bruker 418 s spectrometer at room temperature using a quartz flat cell. The field modulation frequency was 100 kHz. Hyperfine splitting constants and *g* factors were obtained from measurements of magnetic field and microwave

[2] Jordan, W., and Nohl, H. (1983) unpublished observation.

frequency (*8, 9*) made with an AEG nuclear resonance magnetic field meter (AEG-Telefunken, Berlin, West Germany) and a 5340-Å frequency counter from Hewlett–Packard. Paramagnetic adducts of the spin trap DMPO were identified by determining respective coupling constants according to references (*10–13*) and comparing the obtained values with those of the literature (*11–13*).

## RESULTS

### *Reduction of Ubiquinone Derivatives, Menadione, and Platoquinone to Their Respective Stable Semiquinone Radicals*

The quinones under study were dissolved in water-free acetonitrile and reduced by the addition of an $O_2^{\cdot-}$-generating system ($KO_2$ in crown ether). To be sure that all $O_2^{\cdot-}$ radicals produced had been eliminated by reaction with the respective quinones the stoichiometry was adjusted such that quinones were present in excess to trap all unpaired electrons of $O_2^{\cdot-}$ radicals released. The presence or absence of free $O_2^{\cdot-}$ radicals were followed by a spin trapping ESR technique using the DMPO spin trap. The monovalent reduction of the compounds to their respective semiquinone forms was inferred from ESR spectroscopy. Figure 1 shows ESR spectra of the various semiquinone radicals. The $g$ values of these radicals were 2.0048 for menadione and UQ-0, and 2.0049 for UQ-1, UQ-4, UQ-9, and PQ-1. Despite the presence of oxygen, all semiquinone radicals remained stable as long as protons were kept out of the solution. In the presence of protons and oxygen (performed by the addition of small amounts of water) semiquinone-related ESR spectra disappeared (data not shown). These experiments proved that water-free acetonitrile is an appropriate solvent for monovalent electron transfer from $O_2^{\cdot-}$ to quinones, resulting in the generation of stable semiquinone radicals.

### *Reaction of Semiquinone Radicals with $H_2O_2$*

Assuming that $H_2O_2$ molecules have access to the unpaired electrons of these radicals, homolytic cleavage of hydroperoxides is expected to occur, which results in the generation of $OH^{\cdot}$ radicals according to

$$Q^{\cdot} + H_2O_2 \rightarrow Q + OH^{\cdot} + OH^{-}. \qquad [2]$$

This type of reaction was started by the addition of semiquinone radicals dissolved in acetonitrile to a KCl–Hepes buffer, pH 7.4. In addition to $H_2O_2$, the spin trap DMPO was also present to trap any $OH^{\cdot}$ radicals formed. Furthermore, contaminating transition metals which could compete for reaction with $H_2O_2$ were removed from the reaction system by ligating with DETAPAC (see Fig. 3). With the exception of UQ-9 and PQ-1, all the other semiquinones were able to initiate the formation of DMPO–$OH^{\cdot}$-related ESR spectra (Fig. 2a). In the presence of the $OH^{\cdot}$ scavengers ethanol and formate, new types of ESR spectra appeared, indicating the formation of DMPO–hydroxyethyl radical (b), and DMPO–carboxyl radical (c) adducts.

To evaluate the catalytic function of traces of iron possibly contaminating the

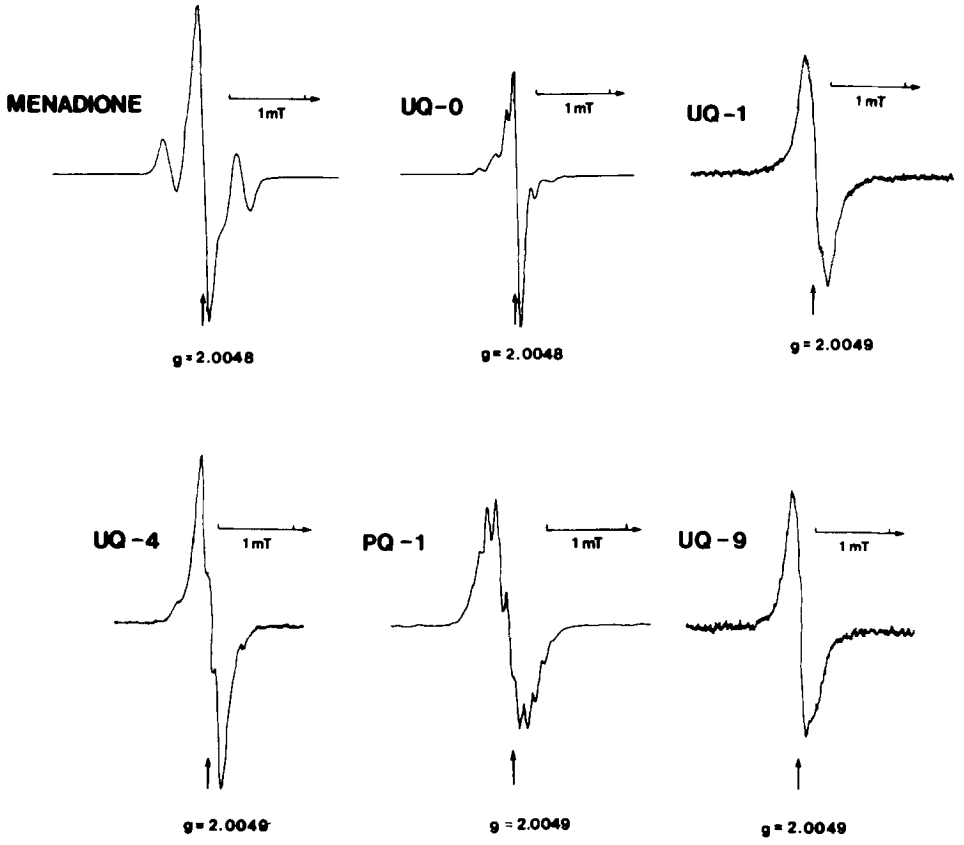


FIG. 1. ESR spectra of semiquinone radicals generated by a monovalent electron transfer from $O_2^-$ in acetonitrile. $O_2^-$ was released from 10 mmol/liter $KO_2$ in 20 mmol/liter crown ether. Final concentration of quinones: 20 mmol/liter; modulation amplitude: 0.04 mT; microwave power: 12 mW: gain for menadione and UQ-0: $1.6 \times 10^3$; gain for PQ-1 and UQ-9: $3.2 \times 10^4$; gain for UQ-1 and UQ-4: $1.25 \times 10^5$.

above reaction system the following experiments were performed. The solvent of Fig. 2 was preincubated with $KO_2$ in crown ether to transform iron ions possibly contaminating the reaction medium to their ferrous state. The existence of ferrous iron was then tested by the addition of $H_2O_2$ to run a Fenton reaction, after having checked that all $O_2^-$ radicals from decomposing $KO_2$ had disappeared (Fig. 3a). However, $OH^\cdot$ radical formation which would indicate a catalytic role of iron in our system could not be detected as long as exogenous iron (Fig. 3b) or quinones (Fig. 3d) were not present in the reaction system. This indicates that impurities of the reaction medium from traces of iron were not critical for $OH^\cdot$ formation from $H_2O_2$. Addition of DETAPAC to chelate iron caused the complete disappearance of $OH^\cdot$-related ESR spectra when added to the Fenton reaction (Fig. 3d). Inhibition of $OH^\cdot$ formation by chelating iron of the Fenton reaction clearly exhibits that iron also complexed to DETAPAC cannot participate in this reaction cycle. To be sure that $OH^\cdot$ radicals detected were not formed by contaminating transition

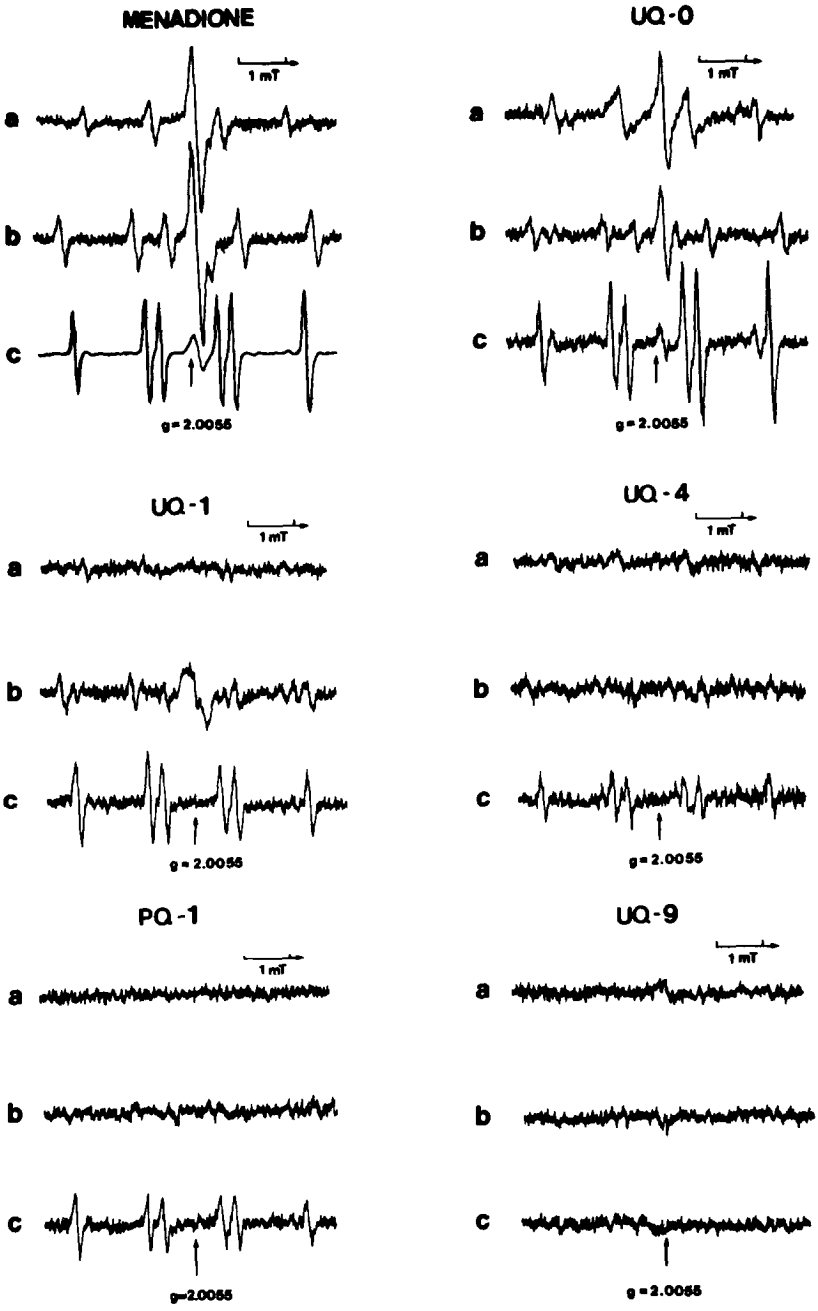


FIG. 2. ESR signals of DMPO spin adducts, obtained by a reaction of semiquinone radicals with 1.5 mmol/liter $H_2O_2$ (a), in the presence of 1 mol/liter ethanol (b) and 1.5 mol/liter formate (c). The reaction was started by mixing semiquinones prepared as described in Fig. 1 with 125 mmol/liter KCl, 50 mmol/liter Hepes buffer, pH 7.4, to give a final mixture of 30 v/v acetonitrile and 70 v/v of water. Final concentration of DMPO: 150 mmol/liters DETAPAC: 1.5 mmol/liter was added to the reaction medium to chelate any contaminating iron. All measurements were performed at room temperature after the removal of oxygen by saturating the reaction medium with nitrogen. Modulation amplitude: 0.04 mT. The assignment of the spectra to the respective adducts of DMPO was made as described under Materials and Methods.

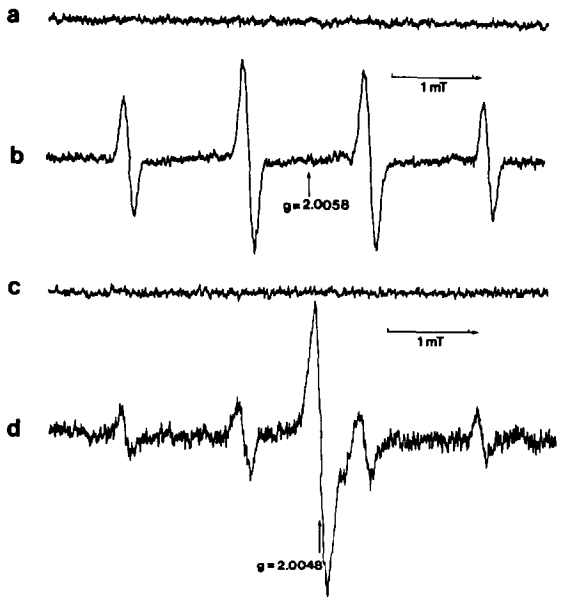


FIG. 3. Effect of possible impurities of the reaction medium of Fig. 2 (a) of exogenous iron (c), and of the quinone menadione (e), on $OH^{\cdot}$ formation in the presence of $O_2^{\cdot-}$ and $H_2O_2$. (b) DETAPAC was added to reaction (a). (d) DETAPAC was added to the Fenton reaction of (c). DETAPAC was also present in reaction (e). $OH^{\cdot}$ generation was evaluated from characteristic ESR spectra of spin adduct formation with DMPO. Final concentration of Fe(III) was 60 $\mu$mol/liter; other reagents and conditions of ESR measurements were identical to those in Figs. 1 and 2.

metals (due to an uncontrolled Fenton reaction) DETAPAC was added to all experiments when the involvement of quinones in $OH^{\cdot}$ formation was investigated.

The existence of overlapping single-line spectra in DMPO-related ESR spectra indicated the presence of semiquinone radicals in reaction systems of menadione, UQ-0, and UQ-1 (Fig. 2). The signal heights of these spectra changed in a manner similar to the signal heights of DMPO spin adducts obtained from the same reaction systems. (Fig. 2b). This observation may indicate the existence of a quantitative correlation between concentrations of semiquinones and the formation of $OH^{\cdot}$ radicals. In order to confirm that the ESR spectra detected in the presence of ethanol and formate represented adduct formation of paramagnetic reaction products from these scavengers with free $OH^{\cdot}$ radicals, we investigated the generation of those spectra in a model system (Fig. 4). $OH^{\cdot}$ generation was performed using a Fenton system (Fe(II)–EDTA, $H_2O_2$) in the same solvent mixture as used before. In the presence of ethanol, a mixed ESR spectrum appeared composed of $OH^{\cdot}$ and the hydroxyethyl radical spin adduct signals (Fig. 4b). Formate caused the appearance of a more prominent DMPO radical adduct spectrum, probably due to higher reaction rates of this scavenger with $OH^{\cdot}$ radicals (*14*) (Fig. 4c). Hyperfine splitting constants for all types of ESR spectra were identical to those obtained from respective reactions with semiquinones under study. Each of these ESR spectra

might therefore be taken as indirect evidence for the formation of free OH· radicals in the presence of semiquinone radicals and $H_2O_2$.

## DISCUSSION

The results presented here support the concept that physiological quinones such as ubiquinone, menadione, and plastoquinone are able to catalyze OH· formation in a reaction analogous to the Haber–Weiss reaction. The detection of semiquinone-related ESR spectra indicated that ubiquinone derivatives, menadione, and plastoquinone undergo a one-electron reduction by an electron transfer from $O_2^{\cdot-}$. The use of an aprotic reaction medium allowed the application of $KO_2$ (in crown ether) as a source for $O_2^{\cdot-}$, and prevented spontaneous disproportionation of the semiquinone radicals formed (*15–17*). Attempts to follow a reaction of these radicals with $H_2O_2$ in acetonitrile by means of spin trapping techniques resulted in the formation of complex spectra with quite similar characteristics, despite the presence of different radical scavengers (data not shown). Distinct ESR spectra which could be assigned to predicted spin adducts required the addition of water to the reaction medium. The concentration of semiquinone radicals in this system is governed by the reaction with $H_2O_2$, and by the spontaneous disproportionation to the hydroquinone and quninone forms. The protonation of the reaction medium favors spontaneous disproportionation (*15*) and might explain the observed decrease in semiquinone-related ESR spectra. The decrease in semiquinone concentrations by the latter pathway should be accelerated in the case of lipophilic quinones (such as UQ-4, UQ-9, and PQ-1) because of the decreased solubility of these compounds in the more protic reaction medium which shifts the reaction to the side of disproportionation.[2] Increased dismutation rates

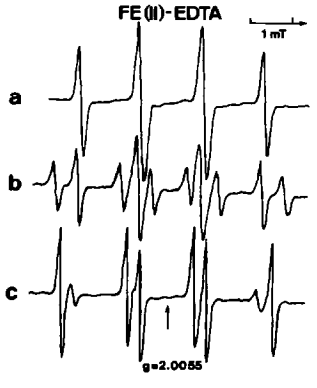


FIG. 4. ESR spectra of DMPO spin adducts obtained by a Fenton-type reaction in the reaction medium of Fig. 2. Final concentrations: DMPO 150 mmol/liter; Fe(II)–EDTA: 300 μmol/liter; $H_2O_2$: 300 μmol/liter (a), ethanol: 700 mmol/liter (b), formate: 1.5 mol/liter (c). Modulation amplitude: 0.04 mT; microwave power: 12 mW.

of semiquinone radicals inhibit $OH^{\cdot}$ formation by a reaction with $H_2O_2$. Consequently, the decrease in semiquinone concentration was found to be paralleled by decreased signal heights of ESR spectra related to adduct formation of $OH^{\cdot}$ or reaction products of this oxygen species (Fig. 2). The absence of any adduct-related ESR signal in the presence of UQ-9 might therefore indicate that semiquinone radicals of this lipophilic compound do not exist in sufficient amounts in the proton-containing reaction system to initiate a reaction with $H_2O_2$. As illustrated by more hydrophilic UQ compounds (UQ-0 and UQ-1, see Fig. 2), the benzoquinone ring which is common to all UQ derivatives undergoes univalent reduction in the presence of $O_2^{\cdot-}$ and oxidation in the presence of $H_2O_2$, finally resulting in the formation of $OH^{\cdot}$ radicals. This observation suggests that UQ-9 when in its natural aprotic environment of the membrane phospholipids can also shuttle electrons to $H_2O_2$ by univalent redox changes. The existence of spin adducts with paramagnetic reaction products of $OH^{\cdot}$ and formate indicate that semiquinone concentrations of PQ-1 and UQ-4 were high enough to initiate a reductive homolytic cleavage of $H_2O_2$. The higher sensitivity of formate for a reaction with $OH^{\cdot}$ as compared to ethanol and DMPO is based on a higher concentration of this scavenger in the reaction medium and higher rate constants for this type of reaction (*14*).

Spectra resulting from $OH^{\cdot}$ radicals and related paramagnetic reaction products could only be obtained from $H_2O_2$ and quinones after their preincubation with $O_2^{\cdot-}$ radicals. This observation strongly supports the idea that semiquinones which have been shown to be generated under our experimental conditions are involved in the formation of $OH^{\cdot}$ radicals from $H_2O_2$ rather than contaminating transition metals (Figs. 3a, 3d). Physiological quinones such as mitochondrial ubiquinone, menadione, and plastoquinone exhibit their biological functions in phospholipid bilayers of biological membranes. Thus, the redox behavior of such quinones in a membrane might be expected to be similar to their behavior in aprotic solvents like acetonitrile. This suggests that semiquinone radicals also participate as intermediates in electron transport systems. The existence of steady-state concentrations of the univalent reduced quinones in the environment of membrane phospholipids has been documented for mitochondrial ubiquinone as an essential intermediate step of electron transfer in the respiratory chain (reviewed by Ohnishi *et al.* (*18*)). Since biological quinones participate in electron translocation, it appears probable that intermediate semiquinone forms of these compounds may also divert electrons from the physiological pathway to $H_2O_2$, giving rise to the formation $OH^{\cdot}$ in the living cell.

$H_2O_2$ which may be regarded as a quasi-physiological by-product of mitochondrial respiration was shown to readily cross the inner mitochondrial membrane from its source of generation in the matrix space to the cytosolic compartment (*19*). The relatively low permeability constant of $H_2O_2$ (*19*) which determines its diffusion across the mitochondrial membrane favors an efficient bimolecular collision with ubisemiquinone radicals, operating as mandatory intermediates of mitochondrial respiration in the same membrane. Thus, such an event yielding steady-state concentrations of $OH^{\cdot}$ radicals has to be considered a realistic process of biological significance.

## ACKNOWLEDGMENTS

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