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Kinetics of Redox Interaction Between Substituted 1,4-Benzoquinones and Ascorbate Under Aerobic Conditions: Critical Phenomena

VITALY A. ROGINSKY[a], TATJANA K. BARSUKOVA[a], GERNOT BRUCHELT[b,*] and HARTMUT B. STEGMANN[c]

[a] *N.N.Semenov Institute of Chemical Physics, Russian Academy of Sciences, Kosygin St. 4, 117977 Moscow, Russia;*
[b] *Children Hospital, University of Tübingen, Rümelinstrasse 23, D-72070 Tübingen, Germany; Institute of Organic Chemistry, University of Tübingen, Auf der Morgenstelle 18, D-72076 Tübingen, Germany*

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Redox cycling is believed to be the most general molecular mechanism of quinone (Q) cytotoxicity. Along with redox cycling induced by a reductase, a similar process is known to occur via electron transfer from ascorbate ($AscH^-$) to Q with formation of a semiquinone radical ($Q^{\bullet-}$):

(1) $Q + AscH^- \xrightarrow{k_1} Q^{\bullet-} + Asc^{\bullet-} + H^+$
(2) $Q^{\bullet-} + O_2 \longrightarrow Q + O_2^{\bullet-}$

The net effect of reactions (1) and (2) provides for the catalytic oxidation of $AscH^-$, with Q serving as a catalyst. In this work, the kinetics of oxygen consumption accompanying this process were studied with several substituted 1,4-benzoquinones (BQ) at 37°C in phosphate buffer, pH 7.40, using the Clark electrode technique. The value of k_1 determined from the initial rate of oxygen consumption was typically found to increase when the one-electron reduction potential $E(Q/Q^{\bullet-})$ shifted to more positive values. With Q, for which $E(Q/Q^{\bullet-})$ is less than −100 mV, the rate of oxygen uptake (R_{OX}) was found to be directly correlated with the $[Q][AscH^-]$ value independent of the concentration of individual reagents, remaining constant for a long period. With mono- and dialkyl-substituted 1,4-BQs, for which $E(Q/Q^{\bullet-})$ is higher than −100 mV, significant deviations from the above simple kinetic regularities were observed. In particular, R_{OX} decreased dramatically with time and critical phenomena (the existence of certain concentrations of Q and/or $AscH^-$ above or below which the catalytic oxidation of $AscH^-$ ceased completely after a non-stationary period of short duration) were observed. These abnormalities can be explained on the basis of the kinetic scheme which contains, in addition to reactions (1)and (2), several side reactions including that between $Q^{\bullet-}$ and $AscH^-$. Implications of critical phenomena discovered in this study for the problems of Q toxicity and vitamin C avitaminosis are discussed.

Keywords: Quinones, ascorbate, redox cycling, critical phenomena, cytotoxicity, catalytic oxidation

Abbreviations: Q, quinone; $Q^{\bullet-}$, semiquinone; QH_2, hydroquinone; 1,4-BQ, 1,4-benzoquinone; $AscH^-$, ascorbate; $Asc^{\bullet-}$, ascorbyl radical; $O_2^{\bullet-}$, superoxide anion radical; R_{OX}, rate of oxygen consumption; $(R_{OX})_0$, starting rate of oxygen consumption; $(R_{OX})_{SS}$, steady-state rate of oxygen consumption; $[Q]_C$ and $[AscH^-]_C$, critical concentrations of Q and $AscH^-$

* Corresponding author. Fax: +49(07071) 294448. E-mail: Gernot.Bruchelt@Uni-Tuebingen.de.

INTRODUCTION

Many quinones (Q) are known as cytotoxic compounds,[1,2] and some of them find application as antitumor agents.[2,3] Among the various molecular mechanisms of Q toxicity suggested in the literature,[1,2] redox cycling is believed to be the most general. It comprises one-electron reduction of Q by NAD(P)H-dependent reductases into semiquinone ($Q^{\bullet-}$), followed by the formation of reactive oxygen species with regeneration of Q due to the reaction of $Q^{\bullet-}$ with molecular oxygen. A similar redox cycling can occur via electron transfer from Q to ascorbate ($AscH^-$) without participation of enzymes:[1]

(1) $Q + AscH^- \xrightarrow{k_1} Q^{\bullet-} + Asc^{\bullet-} + H^+$
(2) $Q^{\bullet-} + O_2 \longrightarrow Q + O_2^{\bullet-}$

The net effect of reactions (1) and (2) provides for the catalytic oxidation of $AscH^-$, with Q serving as a catalyst. This process mimics to some extent an enzymatic redox cycling as both processes have nearly the same elementary steps. In addition, this process is believed to be of more direct biological significance with respect to Q cytotoxicity since many organs are rich in vitamin C (0.6–1.0 mM in brain, liver and pancreas, up to 3 mM in eye lens, adrenals, pituitary gland[4]). Furthermore, the redox interaction of Q with $AscH^-$ may be of pharmacological interest since addition of $AscH^-$ was reported to enhance the efficiency of several quinoid antitumor agents, such as substituted 1,4-benzoquinones (1,4-BQ).[5–8]

Although a possible contribution of Q redox cycling induced by $AscH^-$ to Q toxicity has long been assumed,[1,2,5,6] little is known about the detailed pathways and kinetics of Q–$AscH^-$ interactions. The information obtained until 1991 was summarized in the extensive review,[1] while only a few subsequent studies were reported.[8–12] The activity of Q as a catalyst of $AscH^-$ oxidation was typically characterized by the initial rate of oxygen consumption or hydrogen peroxide formation at certain fixed reagent concentrations. As the process under consideration is initiated by reaction (1), the rate of $AscH^-$ oxidation (R_{OX}) is expected to be proportional to the product of concentration of Q and $AscH^-$

$$R_{OX} = -d[O_2]/dt = -d[AscH^-]/dt = d[O_2^{\bullet-}]/dt = k_{EFF}[Q][AscH^-] \quad (1)$$

where k_{EFF} is in the simplest case equal to the rate constant for the elementary reaction (1), k_1. In turn, the value of k_1 is believed to correlate with a change in one-electron reduction potential in reaction (1):

$$(\Delta E)_1 = E(Q/Q^{\bullet-}) - E(Asc^{\bullet-}/AscH^-) \quad (2)$$

This suggests that the activity of Q as a catalyst increases when $E(Q/Q^{\bullet-})$ becomes more positive. This correlation was really observed,[1] but several Qs with more positive values of $E(Q/Q^{\bullet-})$ did not follow this correlation. The reason for this deviation remains unknown.

In this work, the kinetics of oxygen consumption accompanying redox interaction of $AscH^-$ with several Qs have been studied in detail at variable concentrations of $AscH^-$ and Q. Along with the determination of the initial rate of oxygen consumption, $(R_{OX})_0$, much effort has been made to explore the change in R_{OX} with time. It was found that some mono- and disubstituted 1,4-BQ displayed much more complex kinetic behavior than had been anticipated on the basis of Eq. (1).

MATERIALS AND METHODS

Duroquinone and 9,10-phenanthrenequinone were purchased from Sigma; 2,6-dimethyl-1,4-benzoquinone, 2-methyl-5-isopropyl-1,4-benzoquinone and 1,4-naphthoquinone were from Aldrich; 2,5-dimethyl-1,4-benzoquinone was from Fluka; *tert*-butyl-1,4-benzoquinone was from EGA Chemie; methyl-1,4-benzoquinone was from Merck. 2,6-Diphenyl-1,4-benzoquinone

was gifted by AKZO. Ethyl-1,4-benzoquinone, 2,3-dimethyl-1,4-benzoquinone and 2,3,5-trimethyl-1,4-benzoquinone were prepared by oxidation of the corresponding hydroquinones with $K_3Fe(CN)_6$ in the 1:1 mixture of benzene and diethyl ester. The quinone derivative of 6-hydroxydopamine was obtained via the oxidation of 6-hydroxydopamine (Sigma) in phosphate buffer, pH 7.4, as described in Ref. [11] and used immediately. Ascorbic acid (Fluka) and Chelex-100 resin (Bio-Rad) were used as received. Sodium phosphates, Na_2HPO_4 and NaH_2PO_4, of highest grade were purchased from Merck. All other materials were of highest commercially available grade.

Experiments were performed at 37.0 ± 0.1°C in 50 mM phosphate buffer, pH 7.40 ± 0.02, which was prepared by mixing 50 mM solutions of Na_2HPO_4 and NaH_2PO_4 without adding any acid or base. The solutions of individual phosphates were prepared with double distilled water and purged from traces of transition metals by Chelex-100 resin using the batch method.[13] Stock solutions of Q were prepared with double distilled water or aqueous DMSO, depending on the solubility of Q. Stock solutions of $AscH^-$ (50 mM) were prepared with double distilled water.

The kinetics of oxygen were studied with a 5300 Oxygen Biological Monitor (Yellow Springs Instruments Co., USA) using a Clark electrode as a sensor. The rate of oxygen consumption was calculated from the slope of $[O_2]$ traces. Runs were started with the determination of R_{OX} during the oxidation of $AscH^-$ alone ($(R_{OX})_A$). A typical value of $(R_{OX})_A$ during the oxidation of 1 mM $AscH^-$ was as low as 6–10 nM/s which met the requirements of the Buettner test.[13] Then Q was added from a stock solution without interrupting $[O_2]$ monitoring. The protocol of these experiments was given in more detail in our previous works.[8,9,12] The rates of oxygen consumption presented below were universally corrected for $(R_{OX})_A$. Hence the values of R_{OX} reported here are always the rates of the catalytic process.

Kinetic computer simulations were performed using the program 'Kinetics' (on the base of the Geer method) elaborated by A. Sokolov and I. Utkin.

RESULTS

When reaction (2) is the only path of $Q^{\bullet-}$ transformation and a contribution of other reactions with the participation of $Q^{\bullet-}$ is negligible, an effective regeneration of Q occurs and the steady-state concentration of Q is actually not different from the starting one. As a result, R_{OX} remains constant over a long period. The relation between R_{OX} and the reagent concentrations is given by Eq. (1) where k_{EFF} coincides with the k_1 value. Under these conditions the capability of Q to catalyze $AscH^-$ oxidation is maximized and basically determined by the reduction potential $E(Q/Q^{\bullet-})$. Such a behavior of the Q–$AscH^-$ system is designated as 'standard'. Among the Qs studied, such a 'standard' behavior was shown for the following compounds: 2,3,5-trimethyl-1,4-BQ, duroquinone, 1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone, 9,10-phenanthrenequinone, Q from 6-hydroxydopamine as well as ubiquinone-1 studied earlier[8] (Table I). As seen from Figure 1(A), the rate of $AscH^-$ oxidation catalyzed by the above Q remained practically constant over a long period. In accord with Eq. (1), R_{OX} was proportional to $[Q][AscH^-]$ independently of the concentration of the individual reagents (Figure 1(B)).

In contrast, the behavior of all the studied mono- and dialkyl-substituted 1,4-Q as well as 2,6-diphenyl-1,4-BQ differed noticeably from a 'standard' one (Table I). Several examples are given below.

2,6-Diphenyl-1,4-benzoquinone Equation (1) predicts that, when $[Q][AscH^-]$ is kept constant, the process occurs at the same rate independent of the concentrations of Q and $AscH^-$. However, oxidation of $AscH^-$ in the presence of 2,6-diphenyl-1,4-BQ did not follow this scheme

TABLE I The correlation of the activities of Q as catalysts of $AscH^-$ oxidation with the one-electron reduction potential $E_7(Q/Q^{\bullet-})$. Q activities are given as k_{EFF} determined in this work from $(R_{OX})_0$ by Eq. (1) (fourth column) and R_{OX} reported in Ref. [1] (last column)

Q	Kinetic behavior	$E_7(Q/Q^{\bullet-})$ (mV)[a]	k_{EFF} ($M^{-1}s^{-1}$)	R_{OX}[bb] nM/s
2,3,5,6-$(Me)_4$-1,4-BQ	s	−260	3.5 ± 0.4	18
Q from 6-hydroxydopamine	s	−232[c]	4.8 ± 0.6	—
2-Me-1,4-naphthoquinone	s	−203	3.6 ± 0.6	8.3
2,3,5-$(Me)_3$-1,4-BQ	s	−165	36 ± 5	120
Ubiquinone-1	s	−150[d]	27[9]	—
1,4-Naphthoquinone	s	−140	45 ± 5	150
9,10-Phenathrenequinone	s	−124	85 ± 7	340
2,6-(cyclo-$C_6H_{11})_2$-1,4-BQ	ns	−80[e]	≥ 100[f]	—
2,6-Ph_2-1,4-BQ	ns	—	≥ 300[f]	—
2,6-$(Me)_2$-1,4-BQ	ns	−80	≥ 500[f]	140
2,3-$(Me)_2$-1,4-BQ	ns	−74	≥ 800[f]	—
2-Me-5-i-Pr-1,4-BQ	ns	−70[g]	≥ 400[f]	—
2,5-$(Me)_2$-1,4-BQ	ns	−67	≥ 700[f]	140
2-*tert*-Bu-1,4-BQ	ns	−32[18]	≥ 1600[f]	—
2-Et-1,4-BQ	ns	~ 0[g]	≥ 400[f]	—
2-Me-1,4-BQ	ns	+23	≥ 1200[f]	0.25

's' and 'ns' in the second column mean a 'standard' or 'non-standard' kinetic behavior of Q (see text).
[a] Taken from Ref. [15] unless other indicated.
[b] Reported in Ref. [1] for aerobic interaction of 100 μM Q with 100 μM $AscH^-$ in pH 7.40 buffer at 20°C.
[c] Assumed to be equal to $E(Q/Q^{\bullet-})$ for Q from TOPA.[16]
[d] Assumed to be equal to $E(Q/Q^{\bullet-})$ for coenzyme Q-10.[15]
[e] Assumed to be equal to $E(Q/Q^{\bullet-})$ for 2,6-$(Me)_2$-1,4-BQ.
[f] A maximum value determined.
[g] Estimated from the linear correlation of $E(Q/Q^{\bullet-})$ in acetonitril taken from Ref. [17] with that in aqueous buffer.

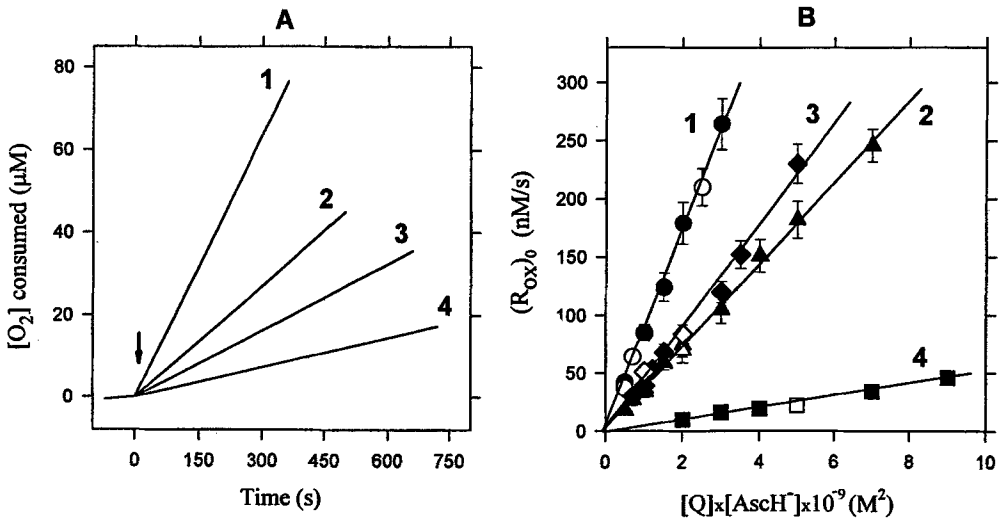

FIGURE 1 Kinetics of $AscH^-$ oxidation catalyzed by 'standard' Q. (A): $[O_2]$ traces observed during the oxidation of 1 mM $AscH^-$ in the presence of 2.5 μM 9,10-phenathrenequinone (trace 1); 2 μM 1,4-naphthoquinone (trace 2); 1.5 μM 2,3,5-trimethyl-1,4-BQ (trace 3) and 5 μM Q from 6-Hydroxydopamine (trace 4). Arrow shows the moment when Q was added. (B): Plots of $(R_{OX})_0$ against the product of [Q] and $[AscH^-]$ at the oxidation of 1 mM (filled symbols) and 0.1 mM $AscH^-$ (hollow symbols) in the presence of 9,10-phenathrenequinone (plot 1); 2,3,5-trimethyl-1,4-BQ (plot 2); 1,4-naphthoquinone (plot 3); Q from 6-hydroxydopamine (plot 4). $(R_{OX})_0$ was calculated as the difference between R_{OX} in the complete reaction mixture and that without Q.

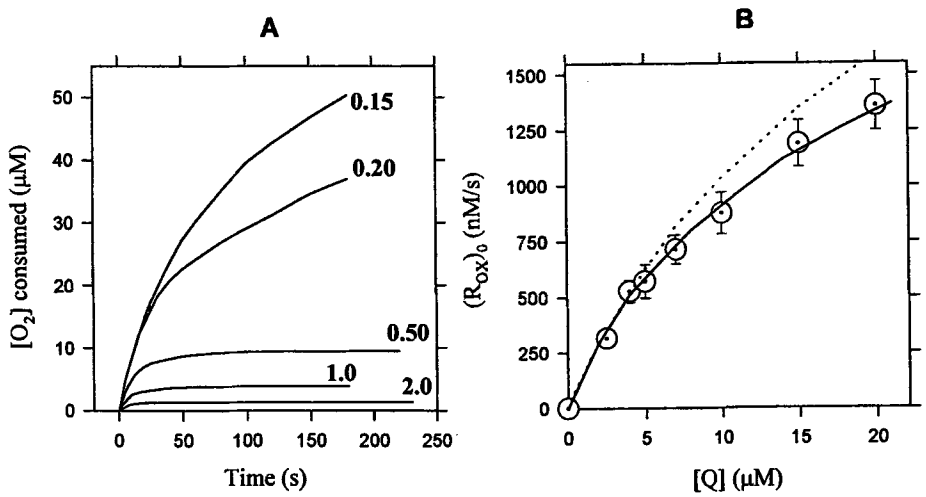

FIGURE 2 Kinetics of $AscH^-$ oxidation in the presence of 2,6-diphenyl-1,4-BQ. (A): $[O_2]$ traces during the oxidation of $AscH^-$ in the presence of 2,6-diphenyl-1,4-BQ at various concentrations of $AscH^-$ (given in mM). In all the runs the $[Q][AscH^-]$ value was kept constant and equal to $3 \cdot 10^{-9}\,M^2$. (B): Plots of $(R_{OX})_0$ against [Q] during the oxidation of 1 mM $AscH^-$ (circles and solid line); dotted line shows this plot simulated with the following parameters (rate constants are given in $M^{-1}s^{-1}$): $k_1 = 400$; $k_{-1} = 1 \cdot 10^7$; $k_2 = 2 \cdot 10^6$; $k_2 = 2 \cdot 10^8$; $2k_3 = 1 \cdot 10^8$; $2k_4 = 2 \cdot 10^5$; $2k_5 = 2 \cdot 10^6$; $k_6 = 5 \cdot 10^4$; $k_7 = 1 \cdot 10^7$; $k_8 = 1 \cdot 10^5$; $k_9 = 1 \cdot 10^5$; $[O_2] = 200\,\mu M$.

(Figure 2(A)). Contrary to what might be expected, $[O_2]$ traces changed dramatically with change in concentrations of Q and $AscH^-$. At $[AscH^-]$ over 0.5 mM, the intense oxygen consumption which was observed at the first moment after mixing the reagents, ceased within 1–2 min almost completely despite the fact that the $AscH^-$ concentration remained actually unchanged. In the runs with 0.2 and 0.15 mM $AscH^-$, the kinetic picture changed significantly–oxygen consumption continued long enough even if at a rate lower than $(R_{OX})_0$. These observations suggest the existence of a critical concentration of $AscH^-$ within the range of 0.2–0.5 mM at which a drastic change of the kinetic mode occurs. Dependencies of $(R_{OX})_0$ on reagent concentrations did not follow Eq. (1) either. As can be seen from Figure 2(B), $(R_{OX})_0$ was not directly with [Q] at constant $[AscH^-]$ as one might expect from Eq. (1).

Tert-butyl-1,4-benzoquinone A very intense oxygen uptake that was observed at the first moment after adding 1.6 μM *tert*-butyl-1,4-BQ to 0.5–2 mM $AscH^-$ almost ceased within a few minutes (Figure 3(A)). When the starting concentration of $AscH^-$ was lower than 0.5 mM, the shape of the $[O_2]$ traces somewhat altered. While R_{OX} decreased dramatically with time, it dropped only to a certain, differed from zero steady-state level, $(R_{OX})_{SS}$, and then remained almost constant (Figure 3(A)). The situation when an increase in the concentration of the oxidation substrate, $AscH^-$, results in the inhibition of its oxidation seems somewhat paradoxical. Moreover, the deviation of the kinetic behavior of the *tert*-butyl-1,4-BQ–$AscH^-$ system from the 'standard' one extends even further. $(R_{OX})_0$ was found to be proportional neither to [Q] at constant $[AscH^-]$ nor to $[AscH^-]$ at constant [Q] as Eq. (1) requires (not shown). The value of k_{EFF} calculated from $(R_{OX})_0$ by Eq. (1) was found to decrease with $[AscH^-]$ (Figure 3(B)). As is evident from the figure, the effectiveness of *tert*-butyl-1,4-BQ as a catalyst of $AscH^-$ oxidation is maximal at the lowest concentrations of $AscH^-$. This holds true for another 'non-standard' Q studied in this work.

2-Methyl-5-isopropyl-1,4-benzoquinone The behavior of this Q was generally similar to that of 2,6-diphenyl-1,4-BQ and *tert*-butyl-1,4-BQ.

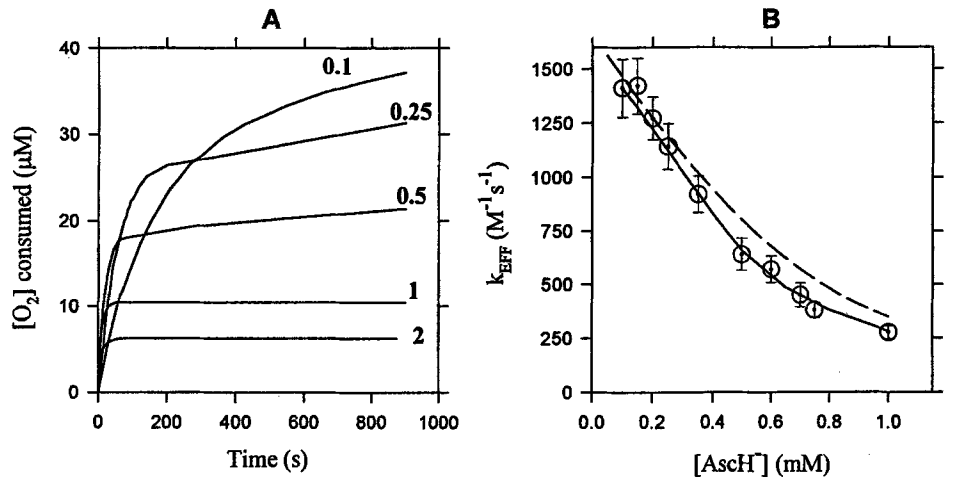

FIGURE 3 Kinetics of $AscH^-$ oxidation in the presence of *tert*-Bu-1,4-BQ. (A): $[O_2]$ traces observed when 1.6 μM Q catalyzes the oxidation of $AscH^-$ taken at various concentrations (indicated in mM). (B): Plot of k_{EFF} determined from $(R_{OX})_0$ by Eq. (1) vs. $[AscH^-]$ at $[Q] = 1.6\,\mu M$ – circles and solid line; dotted line shows this plot simulated at the following parameters (rate constants are given in $M^{-1}s^{-1}$): $k_1 = 4000$; $k_{-2} = 1 \cdot 10^8$; $k_8 = 5 \cdot 10^4$; $k_{-8} = 100$; other parameters as indicated in the legend to Figure 2.

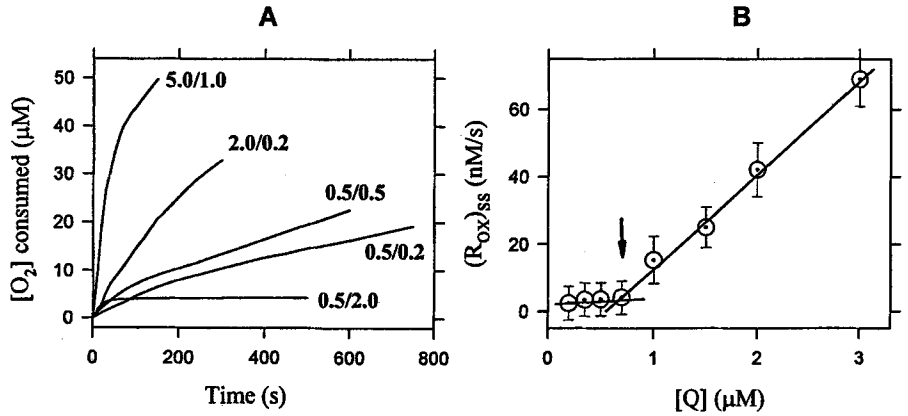

FIGURE 4 Kinetics of $AscH^-$ oxidation in the presence of 2-methyl-5-isopropyl-1,4-BQ. (A): $[O_2]$ traces observed at various concentrations of the reagents – [Q] in μM/$[AscH^-]$ in mM. (B): Plot of the steady-state rate of oxygen consumption, $(R_{OX})_{SS}$, against [Q] during the oxidation of 1 mM $AscH^-$. Arrow shows the critical concentration of Q.

Oxidation of $AscH^-$ ceased or drastically slowed within a few minutes after mixing the reagents (Figure 4(A)). The effect of the decrease of R_{OX} with time became less pronounced when $[AscH^-]$ decreased or [Q] increased (Figure 4(A)). As is evident from Figure 4(B), there is a 'critical' concentration of Q, below which $(R_{OX})_{SS}$ drops down to zero (at about 0.7 μM Q when $[AscH^-]$ is 1 mM). In line with the previous systems, it was possible to suggest a critical concentration of $AscH^-$ above which catalytic oxidation of $AscH^-$ ceases completely after a rather short non-stationary period.

In conclusion, the most characteristic features of the 'non-standard' kinetic behavior of mono- and disubstituted 1,4-BQ are as follows:

- These Qs, taken even at concentrations of ca. 1 μM, induce very fast oxidation of $AscH^-$ at the moment of mixing the reagents which is

followed by a dramatic decrease of R_{OX}. The latter effect becomes more pronounced as [AscH$^-$] increases and [Q] decreases.

- Concentration dependencies of $(R_{OX})_0$ at constant values of [AscH$^-$] and [Q] are not linear, and thus the simple Eq. (1) does not work. The value of k_{EFF} calculated from $(R_{OX})_0$ by Eq. (1) drops down when [AscH$^-$] increases.
- Critical phenomena, observed in this study for the first time, are likely the most noticeable feature of the systems under consideration. They suggest the existence of critical concentration of Q, and that of AscH$^-$, $[AscH^-]_C$ and $[Q]_C$, respectively, below and above which oxidation of AscH$^-$ completely ceases after a rather short non-stationary period ($(R_{OX})_{SS} = 0$).

DISCUSSION AND KINETIC SIMULATIONS

Table I gives the correlation between k_{EFF} and $E(Q/Q^{\bullet-})$. As mentioned above, k_{EFF} calculated from $(R_{OX})_0$ for 'standard' Qs by Eq. (1) is almost equal to k_1. For 'non-standard' Qs, the values of k_{EFF} estimated from $(R_{OX})_0$ by Eq. (1) can be considered as a lower limit of k_1 only, even at the lowest concentrations of AscH$^-$. It can be seen that the values of k_1 determined in this study for 'standard' Q as well as those estimated for 'non-standard' Q correlate reasonably with $E(Q/Q^{\bullet-})$, i.e. k_1 increases when $E(Q/Q^{\bullet-})$ becomes less negative. However, with Q for which $E(Q/Q^{\bullet-}) > -100$ mV, the correlation is less pronounced likely due to some discordance between the estimated and true values of k_1.

The above observations show that the capability of many Q to catalyze the oxidation of AscH$^-$ cannot be unequivocally estimated from $(R_{OX})_0$ measured at any fixed concentrations of Q and AscH$^-$. When $(R_{OX})_0$ is determined at various reagent concentrations, this can result in a change of the position of a given Q in the range of catalytic activity. This may be illustrated by the comparison of values of k_{EFF} determined in this study with R_{OX} reported earlier[1] for the mixture of 100 μM Q and 100 μM AscH$^-$ (Table I). In particular, 9,10-phenathrenequinone and 1,4-naphthoquinone were reported[1] to be more active as compared with methyl- and dimethyl-1,4-BQ, in contrast to the relative activities of Q determined in this study; with methyl-1,4-BQ, the difference is especially impressive. This discrepancy may be explained by that the experiments in work[1] were conducted at very high [Q]/[AscH$^-$] ratio when the deviation from the 'standard' kinetics is expected to be maximal as it follows from our observations (Figures 2–4).

The kinetic properties of 'non-standard' Q may be understood on the basis of Scheme 1. The kinetics of the process under study are highly dependent on $E(Q/Q^{\bullet-})$ due to the control of this thermodynamic parameter over the rate

(1) (−1)	$Q + AscH^- \leftrightarrows Q^{\bullet-} + Asc^{\bullet-} + H^+$	$k_1; k_{-1}$
(2) (−2)	$Q^{\bullet-} + O_2 \leftrightarrows Q + O_2^{\bullet-}$	$k_2; k_{-2}$
(3) (−3)	$Q^{\bullet-} + Q^{\bullet-} + 2H^+ \leftrightarrows Q + QH_2$	$2k_3; k_{-3}$
(4)	$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \longrightarrow O_2 + H_2O_2$	$2k_4$
(5)	$Asc^{\bullet-} + Asc^{\bullet-} \rightarrow AscH^- + A(-H)$	$2k_5$
(6)	$AscH^- + O_2^{\bullet-} + H^+ \longrightarrow Asc^{\bullet-} + H_2O_2$	k_6
(7)	$O_2^{\bullet-} + Q^{\bullet-} + 2H^+ \longrightarrow Q + H_2O_2$	k_7
(8) (−8)	$Q^{\bullet-} + AscH^- + H^+ \leftrightarrows QH_2 + Asc^{\bullet-}$	$k_8; k_{-8}$
(9)	$QH_2 + O_2^{\bullet-} \longrightarrow Q^{\bullet-} + H_2O_2$	k_9

SCHEME 1 Redox interaction of AscH$^-$ with Q under aerobic conditions

constants for the elementary reactions with participation of Q and $Q^{\bullet-}$. Specifically, k_{-2} increases and k_2 decreases when $E(Q/Q^{\bullet-})$ becomes more positive;[1,19] k_1 increases with $E(Q/Q^{\bullet-})$ (this work). Meanwhile, the second reduction potential, $E(Q^{\bullet-}/QH_2)$, tends to increase when $E(Q/Q^{\bullet-})$ becomes more positive.[15,17] The latter suggests that the reactivity of $Q^{\bullet-}$ in reaction (8) increases and that of corresponding QH_2 in reactions (−8) and (9) decreases with shifting $E(Q/Q^{\bullet-})$ to higher values. In conclusion, although the increase of $E(Q/Q^{\bullet-})$ favors in principle a higher catalytic activity of Q due to the increase of k_1, this is accompanied by the decrease of the rate of reaction (2) as well as increase in the rates of reactions (−2) and (8) causing a reduction of the effectiveness of Q as a catalyst.

As is evident from Table I, the dividing line between 'standard' and 'non-standard' Q is located in the vicinity of $E(Q/Q^{\bullet-}) = -100$ mV. When $E(Q/Q^{\bullet-}) < -100$ mV $Q^{\bullet-}$ reacts practically only via reaction (2) and Q displays a 'standard' behavior. At $E(Q/Q^{\bullet-}) > 100$ mV, the contribution of side reactions increases and the process cannot be described by the simple kinetic scheme which includes only reactions (1) and (2). In particular, when k_1 and k_{-2} increase and k_2 drops, the equilibrium (2) shifts to the left; the net result is that a significant part of $Q^{\bullet-}$ decays by reaction (3) transforming into QH_2. Reaction (8) also brings about a decrease in $[Q^{\bullet-}]$ and transformation of Q into QH_2. These two side reactions cause a rather fast reduction of a steady-state concentration of Q with time with the result that redox cycling is suspended. For continuous catalytic oxidation of $AscH^-$, Q regeneration (a reverse transformation of QH_2 into Q) is required. This may occur by reactions (−8) and (9). We conclude that whereas $(R_{OX})_0$ is determined basically by Eq. (1), as for the 'standard' Q, the value of $(R_{OX})_{SS}$ is expected to depend on the effectiveness of Q regeneration as well. However, when Q is converted into QH_2 too fast, even $(R_{OX})_0$ may appear to be lower than the value of $k_1[Q][AscH^-]$.

It is interesting to compare the correlation between Q activity and $E(Q/Q^{\bullet-})$ reported in Ref. [20] for Q reduction by cytochrome P-450 reductase mediated by NADPH and that obtained in this work. They are qualitatively identical, in both cases the correlation is violated when a value of $E(Q/Q^{\bullet-})$ exceeds a certain limit of ca. −100 mV in our work and −165 mV in Ref. [20]. The authors[20] explained this violation by a significant role of the direct redox interaction of Q with NADPH, when $E(Q/Q^{\bullet-}) > -165$ mV. It is not excluded that the reaction between $Q^{\bullet-}$ and NADPH (an analog of reaction (8) in Scheme 1) is the other reason of this effect.

Critical phenomena are likely the most noticeable feature of the systems under consideration. By critical phenomena one means the situation when even small variations of a parameter, for instance, the reagent concentration, temperature, etc., in the proximity to a certain value which essentially differs from zero (a critical point), results in a dramatic change of a function, for instance, steady-state reagent concentrations, rate of reactions, etc. Critical phenomena are widely distributed in chemical and physical branched-chain reactions, they are less known for non-branched free-radical processes.[21] The appearance of critical phenomena in the Q–$AscH^-$–O_2 system is likely due to the double function of $AscH^-$. On the one hand, $AscH^-$ induces the production of $Q^{\bullet-}$ by reaction (1) acting as a prooxidant. On the other hand, $AscH^-$ reacts with chain-carrying free radicals, $O_2^{\bullet-}$ (reaction (6)) and $Q^{\bullet-}$(reaction (8)), acting as an antioxidant. In addition, $AscH^-$ competes with QH_2 for $O_2^{\bullet-}$ hindering the regeneration of Q by reaction (9). Depending on the concentrations of Q and $AscH^-$, one or the other role of $AscH^-$ prevails. When the $[Q]/[AscH^-]$ ratio is high enough, the oxidation of $AscH^-$ may occur in the steady-state mode even if at a rate less than $(R_{OX})_0$. Below a certain (critical) $[Q]/[AscH^-]$ ratio, an effective regeneration of Q becomes impossible, Q is rapidly converted into QH_2 and the catalytic oxidation

of $AscH^-$ ceases completely after a non-stationary period of short duration.

The qualitative agreement of the observed kinetic regularities with Scheme 1 and the reality of critical phenomena, were conformed by results of computer simulations on the basis of Scheme 1. Earlier the related simulations were performed for the case of a standard' Q.[9] It should be noted that the simulations do not pretend to determine the genuine rate constants for elementary reactions. The following rate constants (in $M^{-1}s^{-1}$) reported in the literature at pH 7.4 were used over all the simulations: $2k_4 = 2 \cdot 10^5$;[22] $2k_5 = 2 \cdot 10^6$;[23] $k_6 = 5 \cdot 10^4$.[24] Figures 2(B), 3(B) and 5 give examples of the simulations. Results of simulations reproduce to first approximation the experimentally observed plots of $(R_{OX}A)_0$ vs. [Q] (Figure 2(B)) and k_{EFF} vs. $[AscH^-]$ (Figure 3(B)). The comparison of Figure 5 with Figure 2(A) shows that the simulation is capable to reasonably reproduce the $[O_2]$ traces observed during the oxidation of $AscH^-$ in the presence of 2,6-diphenyl-1,4-BQ and predict the occurrence of the critical concentration of $[AscH^-]$. However, there are no guaranty that the sets of rate constants presented in legends to Figures 2, 3 and 5 were optimum.

The ability of the simulations to reproduce reasonably various experimental data allows to expand the simulations beyond the area of our observations. Among other things, the calculations predict similar critical phenomena when the generation of $Q^{\bullet -}$ via one-electron reduction of Q by a reductase. In this case reaction (0)

$$(0)\quad Q + (\text{reductase}) + NAD(P)H \longrightarrow Q^{\bullet -} + (\text{reductase}) + \text{products} \quad k_0$$

should be used instead of reaction (1) in Scheme 1. Figure 6 demonstrates the appearance of critical phenomena accompanying both the enzymatic redox cycling of Q in the presence of $AscH^-$ and that induced by $AscH^-$. It can be seen that the change from one kinetic mode to the other occurs within a very narrow range of parameters. Figure 6(A1) and 6(B1) show that $[AscH^-]_C$ visibly increases and $[Q]_C$ decreases with the increase in oxygen concentration. Furthermore, both $[AscH^-]_C$ and $[Q]_C$ increase with the increase of the concentration of the

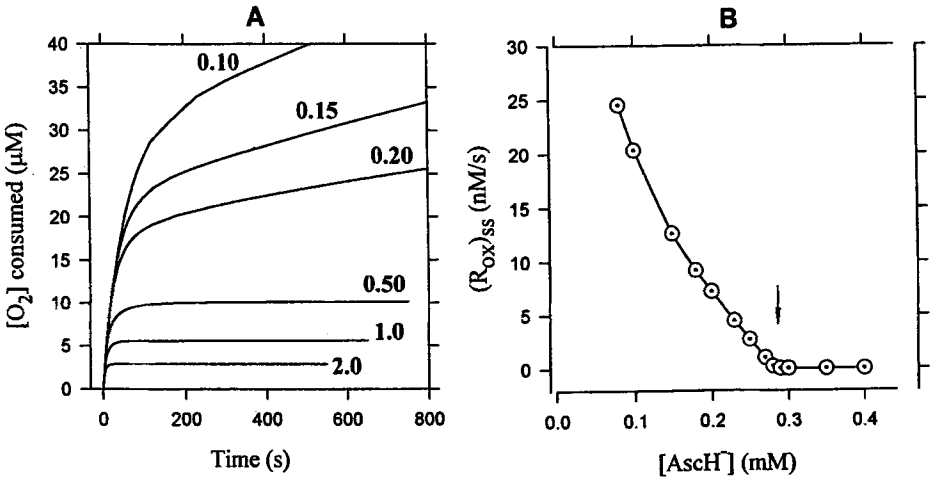

FIGURE 5 Simulation of critical phenomena during the oxidation of $AscH^-$ catalyzed by Q at $[Q][AscH^-] = 3 \cdot 10^{-9} M^2$. Kinetic parameters were as indicated in the legend to Figure 2. (A): simulated $[O_2]$ traces at various concentrations of $AscH^-$ indicated in mM; (B): Plot of the steady-state rate of oxygen uptake against $[AscH^-]$. Arrow shows the calculated critical value of $[AscH^-] = 0.283 \pm 0.002$ mM.

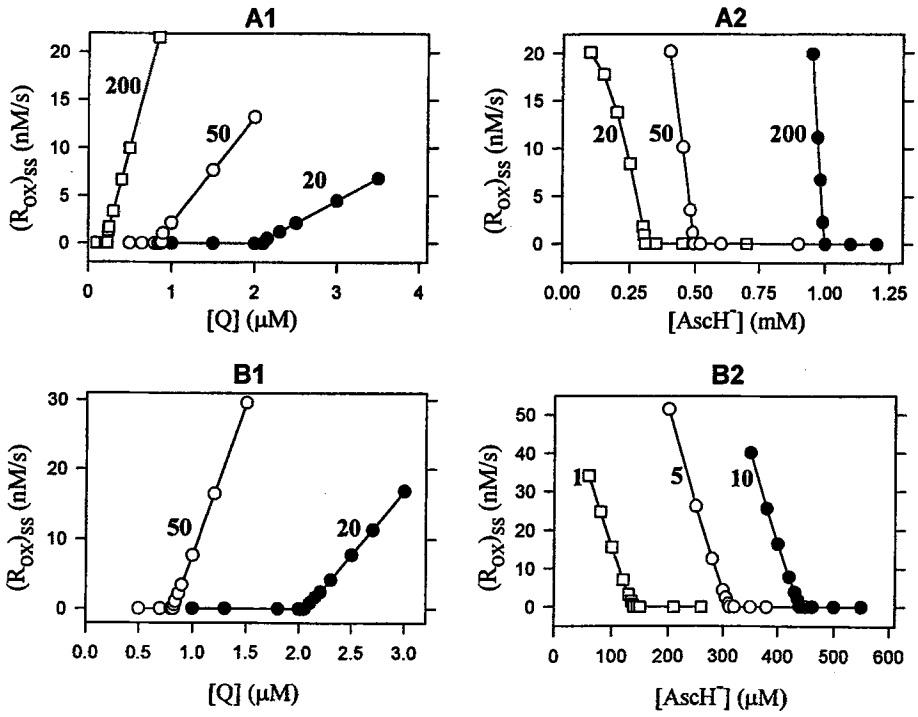

FIGURE 6 Simulation of critical phenomena in Q-AscH$^-$ system. (A1) and (A2) – redox cycling is initiated by reaction (1); (B1) and (B2) – redox cycling is initiated by a reductase (reaction (0)). Kinetic parameters for (A1) and (A2) (rate constants are given in $M^{-1}s^{-1}$): $k_1 = 500$; $k_2 = 1{\cdot}10^7$; $k_{-2} = 1{\cdot}10^8$; other parameters as indicated in the legend to Figure 2. Kinetic parameters for (B1) and (B2) are the same as they were taken in the case of (A1) and (A2) with the exception that $k_1 = 0$, $k_0 = 1{\cdot}10^5\,M^{-1}s^{-1}$ and [reductase] = 5 μM; $[O_2]$ is given in μM. (A1): Plots of $(R_{OX})_{SS}$ against [Q] at $[AscH^-] = 0.2$ mM. (A2): Plots of $(R_{OX})_{SS}$ against $[AscH^-]$ at [Q] = 5 μM. (B1): Plots of $(R_{OX})_{SS}$ against [Q] at $[AscH^-] = 0.2$ mM at various concentrations of O_2 given in μM. (B2): Plots of $(R_{OX})_{SS}$ against $[AscH^-]$ at $[O_2] = 20$ μM and various concentrations of Q given in μM.

second reagent (Figure 6(B2)). The simulation predicts also the existence of critical concentration of oxygen (not shown).

A noticeable peculiarity of enzymatic redox cycling which may be suggested from the simulation, is that the critical concentrations do not actually change with variation of the rate of $Q^{\bullet-}$ formation in reaction (0). For instance, for the system described by the set of parameters given in legend to Figure 6B2 at [Q] = 1 μM, the 10-fold increase in k_0[reductase], from 0.5 to 5 s^{-1}, results only in 1.5%-change of $[AscH^-]_C$ (from 139 to 141 μM). At the same time, the above change in k_0[reductase] causes an pronounced growth of the absolute value of $(R_{OX})_{SS}$ (Figure 6B2).

Calculations also predict that a 'non-standard' behavior of Q and appearance of critical phenomena is also possible in the case of Q with $E(Q/Q^{\bullet-})$ much less than −100 mV though at non-realistically low $[Q]/[AscH^-]$ ratios.

The observations obtained in this study allow some preliminary conclusions of biological significance.

- There is no doubt that the non-enzymatic variant of Q redox-cycling with participation of $AscH^-$ is realistic under *in vivo* conditions and the process under consideration may contribute significantly to Q cytotoxicity, especially in the case of Q with $E(Q/Q^{\bullet-}) > -100$ mV.
- The critical phenomena discovered in this study may be relevant to Q toxicity. In particular, the existence of some critical (boundary) concentration of Q may be predicted, above which Q toxicity increases

dramatically. The values of $[Q]_C$, being dependent on $[O_2]$ and $[AscH^-]$, are expected to be specific for a certain organ. In addition, the existence of a certain critical value of $[AscH^-]_C$ may be anticipated, below which the Q toxicity dramatically increases; this value is likely specific for various organs as well.

- These findings may bear also on the problem of vitamin C avitaminosis. However, our attempts to find any evidence of critical phenomena in biological systems in the literature have been unsuccessful.
- The kinetic peculiarities which describe the Q/$AscH^-$ interactions, specifically critical phenomena, may offer the opportunity to modifiy the action of quinoid anticancer agents.

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References

[1] P.J. O'Brien (1991) Molecular mechanisms of quinone cytotoxicity. *Chemico–Biological Interactions*, **80**, 1–41.
[2] A. Brunmark and E. Cadenas (1989) Redox and addition chemistry of quinoid compounds and its biological implications. *Free Radical Biology and Medicine*, **7**, 435–477.
[3] G. Powis (1989) Free radical formation by antitumor quinones. *Free Radical Biology and Medicine*, **6**, 63–101.
[4] U. Moser (1987) Uptake of ascorbic acid by leukocytes. *Annals of the New York Academy of Sciences*, **48**, 200–215.
[5] R. Pethig, P.R.C. Gascoyne, J.A. McLaughlin and A. Szent-Györgyi (1983) Ascorbate–quinone interactions: Electrochemical, free radical, and cytotoxic properties. *The Proceedings of the National Academy of Sciences of USA*, **80**, 129–132.
[6] P.L. Gutierrez (1989) Mechanism(s) of bioreductive activation. The example of diaziquinone (AZQ). *Free Radical Biology and Medicine*, **6**, 405–445.
[7] C.D. Morgan, S.C. Combs and J. Everse (1995) Probable immune system mediation of the antitumor activity of the 2,6-dimethoxybenzo-p-semiquinone radical. *Proceedings of the Society for Experimental Biology and Medicine*, **208**, 294–299.
[8] V.A. Roginsky, G. Bruchelt and O. Bartuli (1998) 2,3-Dimethoxy-5-methyl-1,4-benzoquinone as effective catalyzer of ascorbate and epinephrine oxidation and dameger of neuroblastoma cells. *Biochemical Pharmacology*, **55**, 85–91.
[9] V.A. Roginsky, D. Mohr and R. Stocker (1996) Reduction of ubiquinone-1 by ascorbic acid is a catalytic and reversible process controlled by the concentration of molecular oxygen. *Redox Report*, **2**, 55–62.
[10] M.C.S. Cano and J.V. Sanchez (1993) Reduction kinetics of 2,3-dimethoxy-5-methyl-1,4-benzoquinone by ascorbic acid in acid solution. *Journal of the Chemical Society. Perkin Transactions*, **2**, 427–430.
[11] S.V. Jovanovic, K. Kónya and J.C. Scaiano (1995) Redox reactions of 3,5-di-tert-butyl-1,2-benzoquinone. Implications for reversal of paper yellowing. *Canadian Journal of Chemistry*, **73**, 1803–1810.
[12] V.A. Roginsky, G. Bruchelt and H.B. Stegmann (1998) Fully reversible redox cycling of 2,6-dimethoxy-1,4-benzoquinone induced by ascorbate. *Biochemistry (Moscow)*, **63**, 200–206.
[13] G.R. Buettner (1988) In the absence of catalytic metals ascorbate does not autoxidize at pH 7: Ascorbate as a test for catalytic metals. *Biochemical and Biophysical Methods*, **16**, 27–40.
[14] V.A. Roginsky, T.K. Barsukova, G. Bruchelt and H.B. Stegmann (1997) The oxidation of catecholamines and 6-hydroxydopamine by molecular oxygen: Effect of ascorbate. *Zeitschrift für Naturforschung. Biological Section*, **52C**, 380–390.
[15] P. Wardman (1989) Reduction potentials of one-electron couples involving free radicals in aqueous solution. *Journal of Physical and Chemical Reference Data*, **18**, 1637–1756.
[16] K. Kano, B. Uno, M. Goto and T. Ikeda (1993) Characterization of TOPA quinone cofactor. *Biochimica et Biophysica Acta*, **1157**, 324–331.
[17] J.Q. Chambers (1974) Electrochemistry of quinones. In *The Chemistry of the Quinoid Compounds* (ed. S. Patai) Part 2, John Wiley and Sons, London, pp. 738–791.
[18] J.K. Dohrmann and B. Berfmann (1995) Equilibria and rates of redox reactions involving the 2-tert-butyl-1,4-benzosemiquinone radical in aqueous solution: An investigation by potentiometry, ESR, and pulse radiolysis. *Journal of Physical Chemistry*, **99**, 1218–1227.
[19] P. Wardman (1990) Bioreductive activation of quinones: redox properties and thiol reactivity. *Free Radical Research Communications*, **8**, 219–229.
[20] J. Butler and B.M. Hoey (1993) The one-electron reduction potential of several substrates can be related to their reduction rates by cytochrome P-450 reductase. *Biochimica et Biophysica Acta*, **1161**, 73–78.
[21] V.A. Belyakov, V.A. Roginsky and N.M. Emanuel (1981) Critical phenomena during peroxide initiator decomposition in the presence of sterically hindered phenols. *Proceedings of the Academy of Sciences of the USSR, Chemistry Section, Translated from Russian*, Vol. 257, pp. 102–105.
[22] B.H.J. Bielski, D.E. Cabelli, R.L. Arudi and A.B. Ross (1985) Reactivity of $HO_2/O_2^{\bullet-}$ radicals in aqueous solution. *Journal of Physical and Chemical Reference Data*, **14**, 1041–1100.
[23] B.H.J. Bielski, A.O. Allen and H.A. Schwarz (1981) Mechanism of disproportionation of ascorbate radicals. *Journal of American Chemical Society*, **103**, 3516–3518.
[24] I.B. Afanas'ev (1989) *Superoxide Ion: Chemistry and Biological Implications*. Volume I, CRC Press, Inc., Boca Raton, Florida.