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The autooxidation of 2,3,5,6-tetrahydroxy-2,5-cyclohexadiene-1,4-dione under physiological conditions

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Summary. A radical anion of 2,3,5,6-tetrahydroxy-2,5-cyclohexadiene-1,4-dione was detected using the EPR technique by complexing with Zn ions. Hydrogen peroxide, superoxide radical anion and hydroxyl radical were also detected in the reaction mixture. Kinetic study and product distribution indicated a probable mixed type one- and two-electron transfer mechanism. A possible relationship between the autooxidation process and the biological activity of substituted quinones was suggested.
Key words. Oxygen activated species; chemiluminescence; quinones; autooxidation; radicals; EPR.

The biological properties of several substances which contain carbonyl groups, such as alloxane, ninhydrin, dihydroascorbate, glyoxal and cyclic ketones are related to the strong reductant activities of these classes of compounds[1,2]. Recently, it has been shown that α-hydroxyketones autooxidize under physiological conditions via the ene-diol tautomer[3,4]. One condition which favors the formation of the ene-diol is the presence of a vicinal carbonyl group (scheme 1).

The equilibrium is in general displaced in favor of the thermodynamically more stable ketol tautomer. The autooxidation of such ene-diols, as well as of ascorbate and dihydrofumarate, has been shown to involve the generation of reactive intermediates such as carbon-centered free radicals, superoxide radical anion (O_2^-), hydroxyl radical (OH·) and hydrogen peroxide[5,6].

One compound which possesses the ene-diol structural element is 2,3,5,6-tetrahydroxy-2,5-cyclohexadiene-1,4-dione (tetrahydroxy-1,4-quinone) (**I**), which bears several structural similarities to coenzyme Q (**II**) (fig. 1) and exhibits a series of biological activities, such as antitrypanosomal[7] and antiviral activities[8,9], and inhibition of glyoxylase I[10], whose selective inhibition could explain carcinostatic activity by preventing metabolism of α-ketoaldehydes in tumor cells. Presumably, all of these effects are related to redox processes[1] and can be reasonably rationalized on the basis of MNDO calculations[11].

These properties led us to study the autooxidation of substituted hydroquinones and seek to establish the relationship between that process and the biological effects induced by these substances. In the present work we report the general features and probable mechanism of quinone **I** autooxidation under physiological conditions.

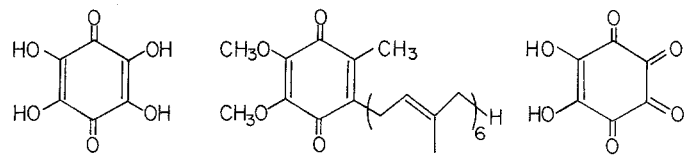
We have observed that the acyl-ene-diol moiety of quinone **I** does indeed undergo autooxidation under physiological conditions with the generation of the intermediate ene-diol radical, superoxide radical anion and hydroxyl radical, the final products being hydrogen peroxide and the corresponding tetrone (**III**) (2,3-dihydroxy-5-cyclohexene-1,2,3,4-tetrone)[12].

Quinone **I** autooxidizes under physiological conditions, as shown by oxygen consumption (fig. 2), with concomitant disappearance of tetrone **III** and appearance of H_2O_2 followed by chemiluminescence method[14] (fig. 3). Superoxide dismutase slightly increased the oxygen uptake, indicating that either oxygen probably reacts very rapidly with one or more of the autooxidation intermediates or an H_2O_2-mediated step in the sequence or the rapid removal of O_2^- displaces the equilibrium of the reaction with a concomitant accelerated O_2 uptake (fig. 3). There is a good correlation between the rate of disappearence of quinone **I** and appearance of the superoxide radical anion as measured by the nitroblue tetrazolium chloride test. The control experiments showed that quinone **I** in the absence of oxygen did not reduce NBT. Presumably the presence of O_2^- and the H_2O_2 appearance simultaneously in this reaction is indicative of a mixed one- and two-electron transfer mechanism[13]. The initial concentration of O_2^- formed in the autooxidation of quinone **I** is low, due to a rapid reaction with the semiquinone to generate H_2O_2 after oxygen consumption (see Eq. 3 in scheme 2). From the data of figure 2, a total of 47 μM of oxygen are consumed by an initial concentration of quinone **I** of 95 μM, indicating an overall stoichiometry of 1:0.5 for the autooxidation (see Eq. 6). The observation of O_2^- and H_2O_2 is indicative of the presence of OH· radicals, and probably singlet oxygen which are part of the Haber-Weiss reaction[15]. The table exhibits data for the influence of various agents on the photon emission during autooxidation of quinone **I** following the method of Durán et al.[16-18]. The

Influence of various agents upon the emission from autooxidation of tetrahydroxy-1,4-quinone[a]

Compounds	Max. intensity counts (× 1000)	Ratio	Remarks
Control	8.7	1.0	—
+ CO_3^{--} (10 mM)	52.9	6.0	Enhancement of the emission when the species are CO_2^-
+ CO_3^{--} (20 mM)	84.7	9.7	Enhancement of the emission when the species are CO_2^-
+ Mannitol (30 mM)	7.3	0.8	OH trap
+ Mannitol (50 mM)	5.2	0.6	OH trap
+ Benzoate (10 mM)	7.0	0.8	OH trap
+ DABCO (10 mM)	39.1	4.5	Enhancement of $^1O_2$ emission
+ Catalase (150 units)	2.0	0.2	H_2O_2 scavenger
+ Catalase denatured (150 units)	5.5	0.6	—
+ SOD (132 units)	2.2	0.3	O_2^- scavenger
+ SOD denatured (132 units)	8.9	1.0	—
+ 1,10-Phenanthroline (10 mM)	1.7	0.2	Complex with iron salts
+ Fe^{++} (10 mM)	57.0	6.6	Free radicals initiator

[a] Tetrahydroxy-1,4 quinone of 1.9 mM in PBS buffer at pH 7.2.



I II III

Figure 1. Chemical structure of quinone I, coenzyme Q (II) and tetrone III.

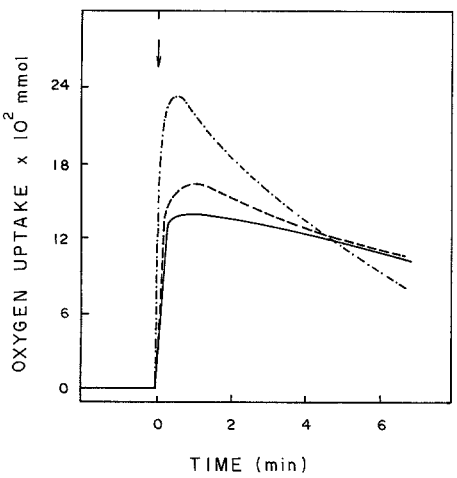


Figure 2. Oxygen uptake measurement in a Clark-type oxygen electrode of quinone I (95 μM) in PBS buffer pH 7.2 (——); after addition of superoxide dismutase (110 units (——) and after addition of catalase (150 units) (–·–·–).

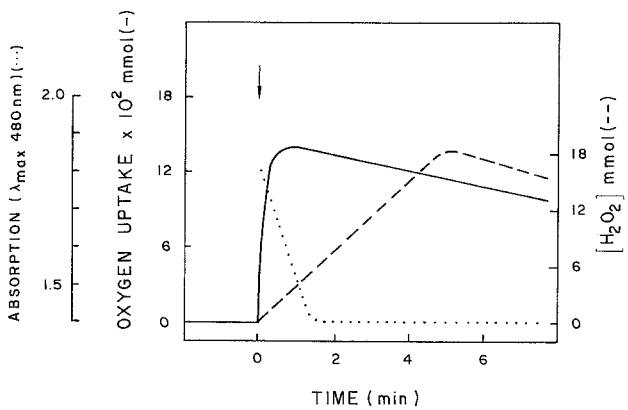


Figure 3. Disappearance of quinone I (...), appearance of H_2O_2 (– – –) and the oxygen uptake (——) at standard conditions for figure 2.

presence of the OH· radical is implied by the decreased emission with mannitol (50 mM) and benzoate (10 mM) and by the strong carbonate effect, ascribed to the reaction of the OH· radical with carbonate to generate the CO_3^- radical[18], which dimerized producing a typical photon emission[16]. DABCO (10 mM) increased the total emission by 4.5-fold, as expected for significant dimol emission from singlet oxygen[16]. The catalase and superoxide dismutase effects are consistent with the presence of H_2O_2 and O_2^- in the reaction mixture. That this chemiluminescence indeed arises from free radical interactions, probably involving a Haber-Weiss reaction, is demonstrated by total quenching of the photon emission in the presence of 1,10-phenanthroline and DETAPAC (not shown), both of which are excellent metal chelating agents. Enhancement of the total photon emission in the presence of iron salt (10 mM) corroborates this conclusion.

Since none of the reagents which were used in these experiments were specific for OH· radical detection the participation of radicals in the quinone I autooxidation was further investigated using EPR spin trapping with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and by direct observation of semiquinone radicals in the presence of Zn^{++}[19]. Figure 4A shows the EPR spectra of quinone I in a Zn^{++} containing buffer. The spectrum is similar to that found for the 1,2-benzenediol radical anion by Klimov et al.[20]. Figure 4B shows the EPR spectra of the OH· generated by a) Fenton's reagent and b) the autooxidation of quinone I in the presence of the spin trap DMPO. Thus, the OH· radical is directly detectable when H_2O_2 reacts with the quinone radical anion[21]. It is known that the formate ion can react with OH· radical to generate the CO_2^- radical[22]. When formate was added to the system in the presence of DMPO, a different spectrum was generated which corresponds to the CO_2^- adduct ($A_N = 15.7$ G and $A_H^\beta = 18.7$ G, not shown).

This is corroborated by the effect of catalase on oxygen consumption shown in figure 2. Oxygen competes with the reaction of H_2O_2 with the radical anion (Eq. 4) through the interaction of oxygen and the dianion (Eq. 2) which generates the OH· radical[3]. It is possible that generation of an excited state of tetrone III may occur by oxidation mediated by an H_2O_2 radical anion, which might develop as a two-step reaction[23,24]. In the presence of catalase, higher concentrations of the radical anion are available for reaction with oxygen due to the elimination of Eq. 4 and this is reflected as greater oxygen uptake. Probably the OH· radical formed from Eq. 4 is important in the photon emission process as shown by the carbonate and mannitol effect. Then in the presence of catalase an inhibition of photon emission was also observed due to the elimination of Eq. 4 which produces OH· radicals.

Taken as a whole, the above data indicate that quinone I autooxidizes to tetrone III (spectral detection and characterization not shown)[1,12], forming an equilibrium mixture whose final quantitative composition is independent of the quinone I initial amount. Figure 5 shows the conversion of quinone I to tetrone III and the opposite reaction followed by absorption spectroscopy. The same effect is observed in oxygen consumption experiments (fig. 6). A sample of 70 μM of quinone I rapidly consumed 35 μM of oxygen; subsequently, the concentration of oxygen in the media increased, stabilizing after a long time at 13 μM. This is also the maximum oxygen uptake at the same concentration of tetrone III, producing probably in a small extension cyclohexanehexone as product. This is indicative of a mainly redox equili-

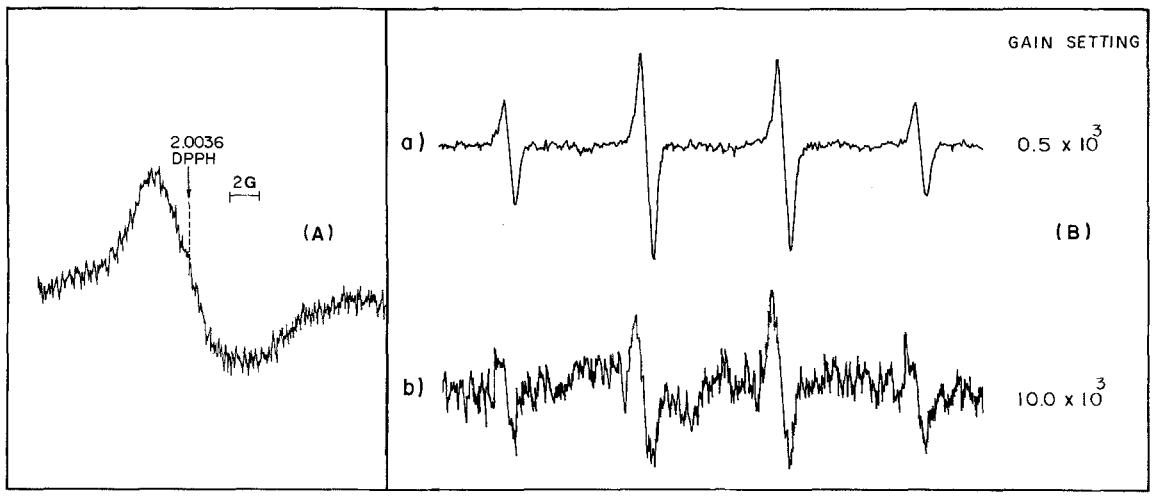


Figure 4. *A* EPR spectra of quinone radical in the presence of Zn^{++} ions (80 mM) and quinone **I** (70 μM) in PBS buffer pH 7.2 at room temperature. *B* a) Spin trapped intermediate with DMPO (150 mM) in the presence of Fenton's reagent and b) from quinone **I** autooxidation. EPR spectra were carried out in a Varian E4 Spectrometer with a field scan of 5 G, modulation frequency 100 kHz, modulation amplitude of 1 G and gain 10,000, and microwave power of 10 mW.

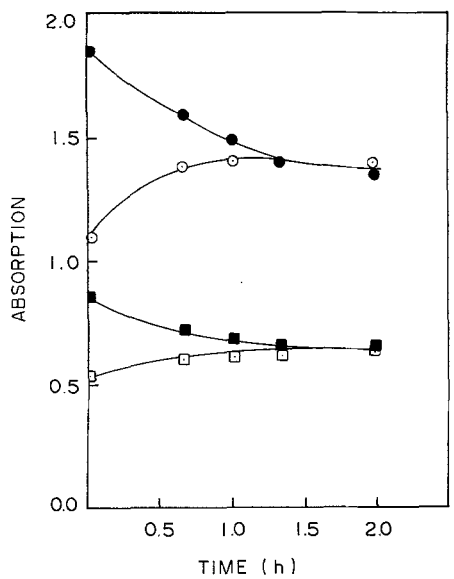


Figure 5. Kinetics of the disappearence of quinone **I** at 480 nm (–●–) and at λ 215 nm (–■–) and appearance of tetrone **III** at 480 nm (–○–) and at 215 nm (–□–) at experimental conditions described in figure 2.

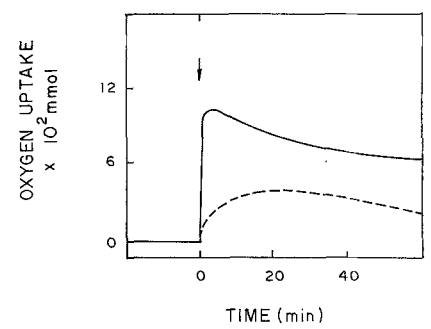
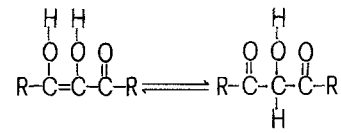


Figure 6. Oxygen uptake measured in a Clark-type oxygen electrode of quinone **I** (70 μM) (——) or tetrone **III** (70 μM) (– – –) in PBS buffer pH 7.2.

brium between quinone **I** and tetrone **III** with an approximate concentration ratio of 1.7.

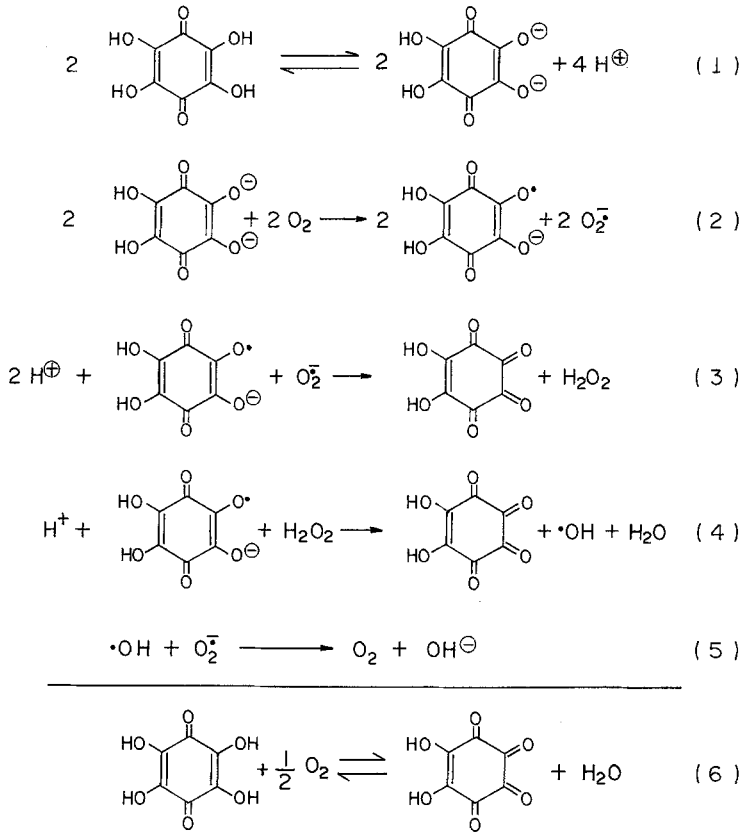
As shown recently by Thornalley et al.[4], ene-diol formation is a prerequisite for autooxidation. In our case, quinone **I** exists completely in the appropriate form and the autooxidation process is probably further activated by remaining features of its molecular structure. Based on the fact that superoxide radical anion, hydrogen peroxide, hydroxyl radicals, singlet oxygen and probably other photoemissive species (e.i. excited quinones) and the quinone radical anion are generated in situ, a mechanism for quinone **I** autooxidation can be tentatively proposed (scheme 2). Dissociation of quinone **I** to the dianion (Eq. 1) and subsequent



Scheme 1

autooxidation the ene-diol moiety (Eq. 2) to the quinone radical anion and the superoxide radical anion accounts for the primary events observed by EPR and NBT reaction. The low concentration of O_2^- observed point to a rapid interaction with the radical anion to produce H_2O_2 and tetrone **III** (Eq. 3). Further reduction of the hydrogen peroxide thus produced by the quinone radical anion to form the OH· radical (Eq. 4) is consistent with the EPR experiments. A possible path for the disappearance of OH· is through equation 5. This is an exothermic interconversion either by 13 kcal/mol ($OH\cdot + O_2^- \rightarrow O_2 + OH$) or by 40 kcal/mol ($OH\cdot + HO_2 \rightarrow H_2O + O_2$), depending on pH conditions[25], but in view of the high reactivity of OH· with the carbon moiety, together with the short lifetime of O_2^- observed, it is unlikely that this process occurs. It is known that these quinones complex metal ions[26] which could catalyze H_2O_2 decomposition to oxygen which could explain its appearance after the rapid consumption of quinone **I** by the autooxidation process. Several products from the overall reaction, in particular the OH· radical and H_2O_2, are potentially toxic to biological systems[27–30].

Thus, it is possible that active oxygen species produced during quinone **I** autooxidation may be involved with its antiviral, antitrypanosomal and antitumor activities.



Scheme 2

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