Generation of OH radical during enzymatic reduction of 9,10-anthraquinone-2-sulphonate

Can semiquinone decompose hydrogen peroxide?

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Spin-trapping experiments have shown that 9,10-anthraquinone-2-sulphonate (AQS) can be reduced enzymatically with NADPH-cytochrome P-450 reductase (FP) producing OH radicals. It has been revealed for the first time that the radical anion of AQS cannot decompose H_2O_2 as indicated by flash-photolysis data. From the experimental results obtained it follows that an enzyme system containing NADPH, AQS and FP produces both accumulation of H_2O_2 from molecular oxygen and reduction of Fe^{3+} to Fe^{2+} which decomposes H_2O_2 catalytically via a Fenton reaction.

Quinone; NADPH-cytochrome P-450 reductase; Hydroxyl radical; Reaction mechanism; Hydrogen peroxide
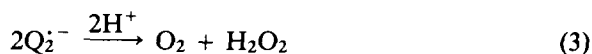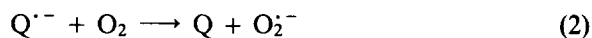
1. INTRODUCTION

Currently quinone-containing antibiotics such as adriamycin, daunorubicin and mytomycin C are widely used in the treatment of various human cancers. It is assumed that the antitumour activity of these compounds is due to their ability to provide redox cycling in cells followed by generation of active forms of oxygen, in particular of highly reactive OH radicals which lead to the destruction of DNA [1–3].

Quinone-containing compounds (Q) are capable of one-electron reduction with different enzymes to produce a semiquinone radical anion [4].

$$Q + e \longrightarrow Q^{\cdot-} \tag{1}$$

Examples of such enzymes are NADPH-cytochrome P-450 reductase [5] and cytochrome P-450 [6–8]. The subsequent rapid auto-oxidation of the radical anion $Q^{\cdot-}$ by oxygen results in the formation of the superoxide radical anion $O_2^{\cdot-}$ and the generation of hydrogen peroxide:

$$Q^{\cdot-} + O_2 \longrightarrow Q + O_2^{\cdot-} \tag{2}$$

$$2O_2^{\cdot-} \xrightarrow{2H^+} O_2 + H_2O_2 \tag{3}$$

As supposed, it is the one-electron reduction of hydrogen peroxide with the radical anion of quinone that produces the OH radical (see e.g. [1,3,9])

$$Q^{\cdot-} + H_2O_2 \xrightarrow{k} Q + OH^- + OH^{\cdot} \tag{4}$$
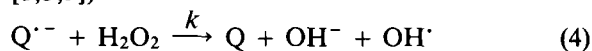
The rate constant of this reaction, as estimated

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using an indirect method for the radical anion adriamycin, ranges from 10^3 to 10^5 $M^{-1} \cdot s^{-1}$ [9].

To study the sequence of reactions leading to formation of the OH radical, we used AQS. This choice is determined by the fact that the photochemical behaviour of AQS in water-alcohol solutions has been studied comprehensively [10]. Upon photoexcitation AQS undergoes one-electron reduction with an ethanol molecule. Note that at neutral pH AQS exists as the radical anion (AQS$^{\cdot -}$) because the pK for AQSH is 3.9 [11]. In the absence of oxygen AQS$^{\cdot -}$ disproportionates to form the initial quinone (AQS) and hydroquinone (AQSH$_2$) [12].

$$2AQS^{\cdot -} \xrightarrow{2H^+} AQS + AQSH_2 \qquad (5)$$
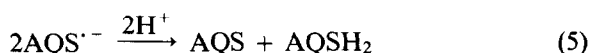
Here, the capacity of AQS for being enzymatically reduced and its capability of generating the OH radical are demonstrated by spin trapping. It is shown for the first ime, via flash photolysis, that the radical forms of AQS cannot effect decomposition of H_2O_2. Based upon the experiments performed, we have assumed that when reduced with FP, AQS stimulates the formation of H_2O_2, on the one hand, and reduces transition metal ions, e.g. Fe^{3+}, on the other, which results in the catalytic decomposition of H_2O_2.

2. MATERIALS AND METHODS

AQS (sodium salt), $Fe_2(SO_4)_3 \cdot 9H_2O$, glucose, glucose oxidase and DMSO (Sojuzkhimreactiv), EDTA, NADPH (Reanal), catalase (Serva), superoxide dismutase (Boehringer Mannheim) and DMPO (Sigma) were purchased from the sources mentioned. Prior to use, DMPO was purified as in [13]. FP, isolated from livers of Wistar rats and purified as in [14], was kindly donated by Dr V.V. Lyakhovich (Institute of Clinical and Experimental Medicine, Novosibirsk). FP activity was determined as described [15]. Formation of the OH radical was judged from the ESR spectra of DMPO registered using an ER 200D-SRC spectrometer (Bruker). A flat cell ($V = 200 \mu l$) was used. Conditions for recording ESR spectra were: microwave frequency, 9.78 GHz; power, 19.8 mW; modulation amplitude, 1.0 G. Photolysis of AQS under stationary conditions was carried out

according to [16]. The kinetics of decay of AQS$^{\cdot -}$ were studied using a flash-photolysis system with a flash energy of 100 J and a duration of 2 μS [16]. The cell (25 ml volume) was 16 cm in length. To prevent photolysis of H_2O_2, BS-6 filters ($\lambda = 340$ nm) were placed between the flashlamp and the cell.

3. RESULTS AND DISCUSSION

In the absence of oxygen, the only route for the decay of AQS$^{\cdot -}$ is its disproportionation via reaction 5. Photoreduction of AQS results in quantitative formation of AQSH$_2$. Fig.1 illustrates the optical absorption spectra of AQS and AQSH$_2$ produced photochemically. The same spectral changes are caused by the NADPH-dependent reduction of AQS with FP under anaerobic conditions.

In the presence of O_2 in the system containing AQS, NADPH and FP, the formation of spectrally detectable AQSH$_2$ was not observed. This is accounted for by the fast reoxidation of AQS$^{\cdot -}$ due to the oxygen present in air (reaction 2). However, under the same conditions oxidation of NADPH took place that was dependent on the concentration of AQS ($K_m = 3.6 \times 10^{-4}$ M). H_2O_2, resulting from reaction 3, can react with AQS$^{\cdot -}$, via reaction 4, to produce the OH radical. To register the formation of the hydroxyl radical, we used DMPO. Finkelstein et al. [17] have indicated possible artifacts during the study of ESR signals of DMPO-OH. For this reason we dissolved AQS in DMSO prior to its introduction into the reaction mixture (final concentration of DMSO, 5%). In this system the primary OH radical reacts with DMSO producing the secondary CH$_3$ radical [18], the interaction of which with DMPO gives rise to the spin adduct that is easily registered by ESR. The presence of the DMPO-CH$_3$ spin adduct is direct evidence that the OH radical is present in the system, since DMSO can only be oxidized to the CH$_3$ radical by very strong oxidants.

The spectrum and kinetics of DMPO-$\cdot$CH$_3$ formation are illustrated in fig.2. In the absence of AQS, no ESR signal was detected. The characteristic feature of the kinetics is the existence of a lag period. It can be associated with the disappearance of oxygen upon its reduction to H_2O_2 (see
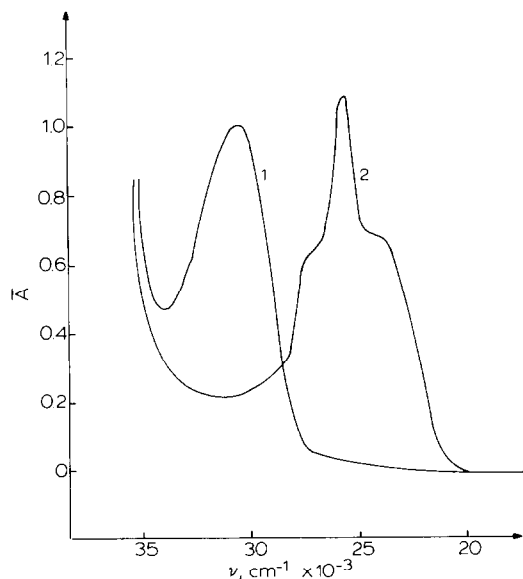
Fig.1. UV spectra of AQS (1) and $AQSH_2$ (2) in a water-ethanol mixture (1:1). $AQSH_2$ was produced by photochemical reduction. Time of irradiation, 1 min; concentration of AQS, 10^{-4} M.

eqns 2,3). This assumption is confirmed by the following experiments. On decrease of the initial concentration of dissolved O_2, the lag period became shorter (fig.2). The same effect was observed when the concentration of reductase in the system increased. At the same reactant concentrations the kinetics of NADPH oxidation attained a plateau after ~10 min, which corresponds to the end of the lag period. Note that by this moment of time, the amount of oxidized NADPH corresponded to the initial amount of oxygen dissolved in the system (~2 × 10^{-4} M) (data to be shown elsewhere).

The addition of SOD to the system had no effect on the kinetics of accumulation of the spin adduct, whereas in the presence of catalase we failed to observe ESR signals. These data demonstrate that H_2O_2 participates in the process under consideration, and that O_2^- makes an insignificant contribution.

To verify the possibility of the occurrence of reaction 4, we have studied the decay kinetics of $AQS^{·-}$ employing the flash-photolysis method in both the presence and absence of H_2O_2. The spectrum of $AQS^{·-}$ generated by a light flash is shown

in fig.3a and is similar to the published spectrum of the anion radical of anthraquinone [10]. In the absence of H_2O_2, $AQS^{·-}$ decomposes, the characteristic time for this process being ~0.5 s. The addition of H_2O_2 up to a concentration of 0.1 M did not affect the kinetics of decay of $AQS^{·-}$ (fig.3b). Taking into account the accuracy in the measurements of the lifetime of $AQS^{·-}$ (~5%, fig.3b), one can state that the bimolecular rate constant of the $AQS^{·-}$ reaction with H_2O_2 is $\leqslant 1$ $M^{-1} \cdot s^{-1}$ and, thus, reaction 4 does not play a significant role in the process of OH radical formation. It should be noted that we failed to observe any changes in $AQS^{·-}$ decay kinetics in the presence of H_2O_2 upon repetition (15–20 times) of the flash photolysis experiments with the same sample. This confirms the conclusion that reaction 4 is insignificant in the generation of OH radicals. Indeed, the OH radical, generated via reaction 4, while interacting with H_2O_2 leads to the formation of O_2^- and later one, according to reaction 3, to oxygen. Accumulation of O_2 in a sample is certain to have a substantial effect on the $AQS^{·-}$ decay kinetics, which was not observed experimentally (fig.3). This led to the conclusion that feasible reactions connected with OH radical participation such as

$$OH^· + C_2H_5OH \longrightarrow H_2O + {}^·CH_3CHOH$$

$$OH^· + QH_2 \longrightarrow H_2O + Q^{·-} + H^+,$$
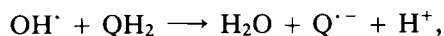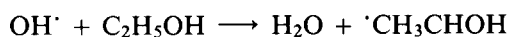
in flash-photolysis experiments may be neglected.

However, as follows from our experiments (fig.2) and from numerous literature data [1,3,9], the reduced forms of quinones stimulate formation of the OH radical. A possible catalyst of H_2O_2 decomposition in these systems could be iron which is present in trace amounts in the system (Fenton reaction) [19], i.e.

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^· \qquad (6)$$
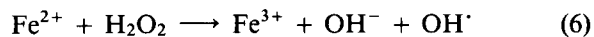
To elucidate the effect of iron traces on formation of the OH radical, we have examined the influence of a water-soluble chelate, viz. iron-EDTA, on the accumulation of the spin adduct (fig.2). The influence was two-fold: the maximum amplitude of the signal, A_{max}, and the characteristic time of decay of the ESR signal of the spin adduct after attaining A_{max} had a tendency to change. With increasing concentration of iron-
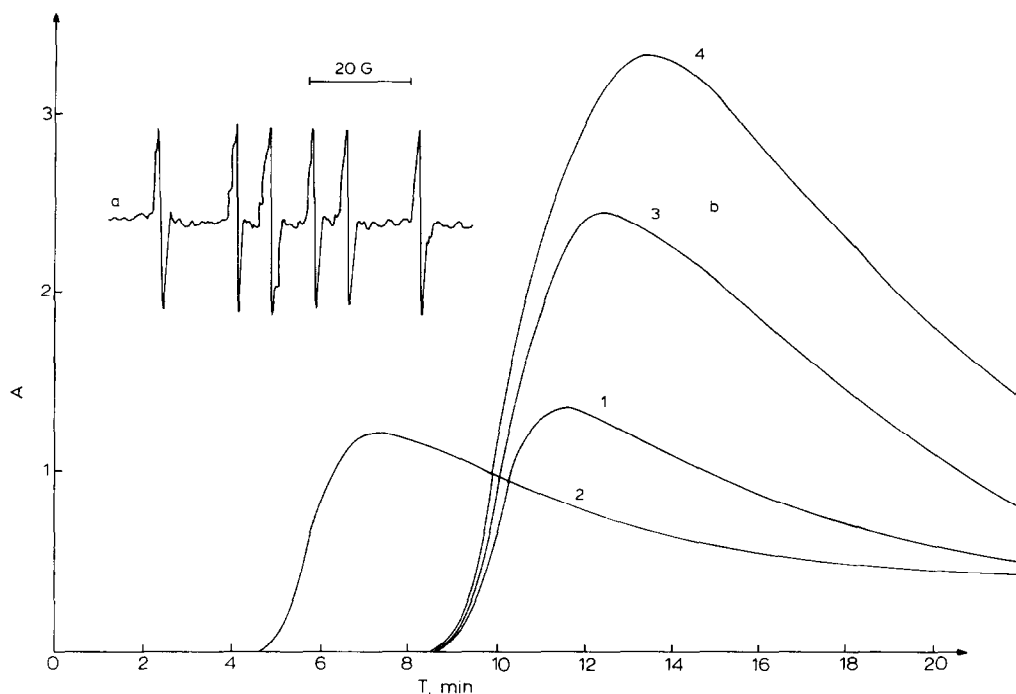
Fig.2. (a) ESR spectrum of the $\cdot CH_3$-DMPO spin-adduct formed in a system containing 10^{-3} M NADPH, 0.3 IU/ml FP, 5×10^{-4} M AQS, 0.1 M DMPO in 0.1 M potassium phosphate buffer (pH 7.6). The solution was introduced into a hermetically sealed flat cell. The spectrum was registered at the moment the amplitude attained its maximum. (b) Accumulation kinetics of the ESR spin adduct $\cdot CH_3$-DMPO measured on the low-field component (a). Experimental conditions and reaction mixture as in panel a. 1, without admixture; 2, a priori the solution was aspirated off using a mixture of air-argon (1:1); 3,4, in the presence of 2.5×10^{-7} M Fe^{3+}-EDTA and 5×10^{-7} M Fe^{3+}-EDTA.
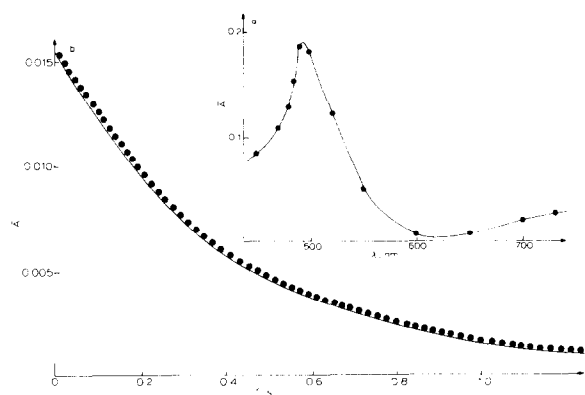

Fig.3. (a) Optical absorption spectrum of $AQS^{\cdot-}$ in water-alcohol mixture (1:1) obtained by pulse excitation. Initial concentration of AQS, 5×10^{-5} M. (b) Kinetic curve for the decrease in $AQS^{\cdot-}$ absorption at 490 nm: (——) without admixtures; ($\cdots$) in the presence of 0.1 M H_2O_2.

EDTA, A_{max} first increases and then starts to decrease (fig.4), which reflects the effect of iron on the decay of the spin adduct.

By approximating A_{max} to zero (fig.4), we have succeeded in estimating the content of impurities of heavy metals in the initial mixture ($\sim 2 \times 10^{-7}$ M).

For reaction 6 to occur, it is necessary for Fe^{3+} to be reduced to Fe^{2+}. In our system this reduction can be achieved in two ways: (i) directly with FP and (ii) with $AQS^{\cdot-}$ via the reaction:

$$AQS^{\cdot-} + Fe^{3+} \longrightarrow AQS + Fe^{2+} \qquad (7)$$

The possibility of reduction of iron-EDTA with FP has been reported in [20]. Using the data of this work we have calculated the rate of reduction for our experimental conditions (see, e.g. the legend to fig.2). The rate was found to be some 100-times slower than that of AQS reduction with FP. Sup-
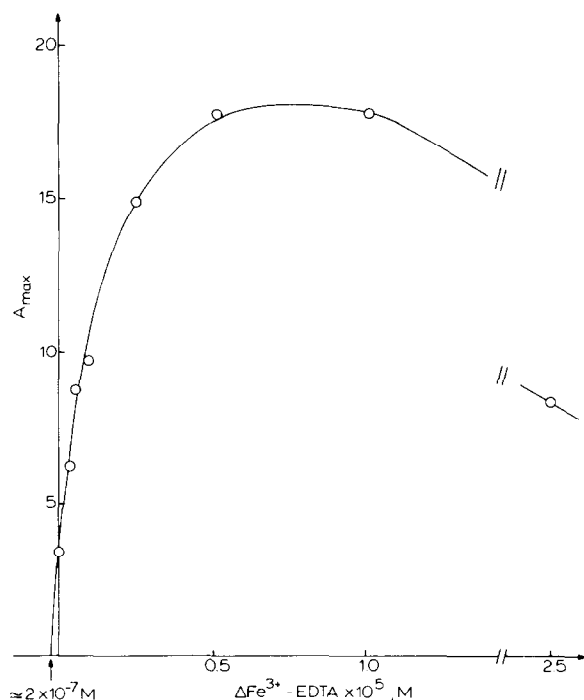
Fig.4. Dependence of maximum ESR signal amplitude (in time) A_{max} of the spin adduct CH_3-DMPO (see legend to fig.2, panel b) on the concentration of Fe^{3+}-EDTA added.

port for the assumption that the reduction of Fe^{3+} to Fe^{2+} occurs exclusively via reaction 7 was provided by our experiments during which the spin adduct was not formed in the anaerobic system containing NADPH, FP, H_2O_2 and iron-EDTA (anaerobiosis and generation of H_2O_2 were accomplished by introduction of glucose and glucose oxidase into the system).

The rate constant of reaction 7 in our system can be rather high (e.g. 4.0×10^7 $M^{-1} \cdot s^{-1}$ for reaction of AQS·⁻ with Cu^{2+} [12]). It should be noted that the redox potential (E_0) for AQS/AQS·⁻ equals -0.380 V [21] and that for Fe^{3+}-EDTA/Fe^{2+}-EDTA is equal to 0.12 V [22], therefore reaction 7 is thermodynamically possible.

The experiments performed allow us to conclude that in the system containing quinone, FP, NADPH and traces of heavy metals, quinone is necessary for the formation of H_2O_2 from dissolved oxygen, and the reduction of Me^{n+} to

$Me^{(n-1)+}$ as well as reaction 7. In view of the fact that AQS·⁻ shows good reductive ability reaction 4 seems to be improbable for other quinones as well, including quinone-containing antitumour antibiotics.

Considering the fairly high rate constants for the reduction of molecular oxygen and the iron-EDTA complex by semiquinones of adriamycin and mytomicin C [23], it is expected that the proposed mechanism of generation of OH radicals is valid for these natural quinones in microsomes and mitochondria. (In preparing the present paper results have been obtained by our group indicating that the proposed mechanism of generation of OH radicals holds for the main antitumour antibiotics adriamycin, daunorubicin and mytomycin C both in a full microsomal system and in the presence of purified NADPH-cytochrome P-450 reductase [to be published later].)

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