Role of Quinone–Iron(III) Interaction in NADPH-Dependent Enzymatic Generation of Hydroxyl Radicals†

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ABSTRACT: To study the effect of chelation of iron ions by quinones on the generation of OH radicals in biological redox systems, we have synthesized quinones that can form complexes with Fe(III) ions: 2-phenyl-4-(butylamino)naphtho[2,3-*h*]quinoline-7,12-dione (Q_c^b) and 2-phenyl-4-(octylamino)naphtho[2,3-*h*]quinoline-7,12-dione (Q_c^o). A quinone with a similar structure without chelating group was synthesized as a control sample: 2-phenyl-5-nitronaphtho[2,3-*g*]indole-6,11-dione (Q_n). Using optical spectroscopy, we determined the stability constant of Q_c^b with Fe(III) [$K_s = (7 \pm 1) \times 10^{18}$ M^{-3}] and the stoichiometry of the complex $Fe(Q_c^b)_3$ in chloroform solutions. One-electron reduction potentials of Q_c^b, Q_n, and adriamycin in dimethyl sulfoxide were measured by cyclic voltammetry. In the presence of Fe(III) the one-electron reduction potentials shifted toward positive values by 0.16 and 0.1 V for Q_c^b and adriamycin, respectively. Using the spin trap 5,5′-dimethyl-1-pyroline *N*-oxide (DMPO) and EPR, it was found that Q_c^b in the Fe(III) complex stimulated the formation of OH radicals in the enzymatic system consisting of NADPH and NADPH–cytochrome P-450 reductase more efficiently than adriamycin and quinone Q_n. This is indicated by the absence of a lag period in the spin adduct appearance for Q_c^b and by a significantly higher rate of the spin adduct production, as well as by a larger absolute concentration of the spin adduct obtained for Q_c^b in comparison with Q_n in the presence of Fe(III). The results obtained allow one to explain the efficient stimulation of oxygen radicals by Q_c^b in the presence of Fe(III) in enzymatic systems by intramolecular electron transfer in the semiquinone of the Q_c^b – Fe(III) complex.

Quinones with antitumor activity were selected from a great number of natural (Thompson, 1971) and synthetic quinones (Thomas, 1974). The characteristic feature of some quinones is their ability to be reversibly oxidized–reduced in aerobic conditions. This results in the formation of semiquinones and oxygen radicals (Powis, 1989) both in electron-carrying chains: microsomes, mitochondria, etc. (Bachur et al., 1978; Doroshow & Davis, 1986), and in cells (Doroshow, 1986; Sinha et al., 1987). Of course, these processes can also occur in simpler systems where electron sources are NAD(P)H-dependent flavoproteins and cofactors are NADH or NADPH (Yamazaki, 1977). The sequence of reactions taking place in electron-carrying chains in the presence of quinones, Q, leading to OH radical generation can be presented as

$$Q + e \xrightarrow{k_1} Q^{\bullet -} \quad (1)$$

$$Q^{\bullet -} + O_2 \xrightarrow{k_2} O_2^{\bullet -} + Q \quad (2)$$

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \xrightarrow{k_3} H_2O_2 + O_2 \quad (3)$$

$$Q^{\bullet -} + Fe(III) \xrightarrow{k_4} Q + Fe(II) \quad (4)$$

$$H_2O_2 + Fe(II) \xrightarrow{k_5} OH^- + {}^{\bullet}OH + Fe(III) \quad (5)$$

Obviously, reactions 1–5 are valid only for quinones with the proper redox properties, i.e., for those that can oxidize reduced enzymes and then reduce molecular oxygen and Fe(III) ions or iron complexes.

It is known that OH radical, one of the most powerful oxidants in chemistry, can cause the single- and double-stranded breaks of DNA (Berlin & Haseltine, 1981; Brawn & Fridovich, 1981) and initiate the peroxide oxidation of lipids (Bisby et al., 1982), etc. It was found that resistance of some lines of tumor cells to adriamycin (Adr) depends on the increased activity of enzymes which decompose hydrogen peroxide and superoxide radicals in cells (Sinha et al., 1987; McGrath et al., 1989; Yin et al., 1989). Thus, it might be concluded that hydroxyl radical generation is an important precondition for the manifestation of antitumor activity of quinone antibiotics. As follows from reactions 1–5, for the generation of OH radicals, the ions Fe(III), Cu(II), etc., or their complexes with organic ligands, which also undergo cyclic oxidation–reduction in electron-transfer chains, are necessary. It has been shown [see Powis (1989) and references therein] that adriamycin and daunomycin form complexes with Fe(III), which can be active in reactions of oxygen radical formation.

We have already reported that synthetic naphthoquinone can participate in reactions 1–5 in the presence of NADPH–cytochrome P-450 reductase and can induce the NADPH-dependent scission of the DNA of plasmide pBR 332 as efficiently as adriamycin (Rumyantseva et al., 1989). In this case $Fe(EDTA)_2$ complexes stimulate both formation of OH radicals and DNA scission. Based on this fact and on the known features of Adr–Fe(III) complexes, it is possible to suppose that quinones capable of chelating metal ions, on the one hand, and providing reactions 1–5, on the other, will be

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more efficient OH radicals generators than quinones having no such features.

In this work we have synthesized quinones that can provide chelation of Fe(III) and those that cannot. It is shown that, in fact, the "chelating" quinone forms the complex with Fe(III). One-electron reaction potentials of these quinones have been measured, and their capability of generating OH radicals in enzymatic systems [both in Fe(III) complex and alone] has been studied by the spin-trap technique.

MATERIALS AND METHODS

Materials. Adriamycin hydrochloride, 5,5′-dimethyl-1-pyroline *N*-oxide (DMPO), NADPH, and cytochrome *c* were obtained from Sigma (St. Louis, MO). Ferric chloride and dimethyl sulfoxide (DMSO) were obtained from Merck (Darmstadt). An aqueous solution of ferric chloride was used for 1 h after preparation. To remove water traces, DMSO was distilled in vacuum and then stored on activated molecular sieves (4 Å). NADPH–cytochrome P-450 reductase was isolated from the liver of male Wistar rats (Dignam & Strobel, 1977) and kindly supplied by Dr. V. Lyakhovich (Institute of Clinical and Experimental Medicine, Novosibirsk). The purified NADPH–cytochrome P-450 reductase preparation has a specific activity of 30 units/mg of protein. One unit of reductase activity is defined as the amount of enzyme catalyzing the reduction of cytochrome *c* with the initial rate of 1 μM/min at 22 °C under described conditions (Phillips & Langdom, 1962).

Quality Control. A spin trap DMPO was purified (Buettner & Oberley, 1978). The absence of paramagnetic admixtures was determined from the EPR spectrum.

Measurements of the NADPH Oxidation Rate. The rate of NADPH oxidation by NADPH cytochrome P-450 reductase was determined, using spectrophotometry, from the decrease in NADPH optical density at λ = 340 nm; ϵ_{340} = 6.22×10^3 M^{-1} cm^{-1}. The reaction mixture was 0.05 units/mL NADPH–cytochrome P-450 reductase, 1 mM NADPH, 10 μM quinone, and 20 mM sodium phosphate buffer (0.2 M NaCl, 1 mM $MgCl_2$, pH = 7.4). Cuvettes with an optical path of 0.1 cm were used.

EPR Study of Radical Formation. OH radical formation was followed by EPR of the spin adduct, DMPO–$^{\bullet}CH_3$, formed under the action of the OH radical in 10% DMSO (Finkelstein et al., 1980; Klein et al., 1981).

$$^{\bullet}OH + CH_3S(O)CH_3 \rightarrow {}^{\bullet}CH_3 + HOS(O)CH_3$$

$$^{\bullet}CH_3 + DMPO \rightarrow DMPO\text{–}{}^{\bullet}CH_3$$

The EPR signal of DMPO–$^{\bullet}CH_3$ is a sextet with hyperfine splitting constants a_N = 15.6 G and a_H = 22.6 G (Finkelstein et al., 1980).

The artifacts that occur in testing OH radicals by DMPO are absent in the case of generation of the spin adduct DMPO–$^{\bullet}CH_3$ (Finkelstein et al., 1980). In experiments on hydroxyl radical generation, the sample contained NADPH–cytochrome P-450 reductase (activity being 0.02 unit), 1 mM NADPH, 10% DMSO, 50 mM DMPO, quinone at wanted concentration in 50 mM Tris-HCl buffer, and 0.2 M NaCl, pH = 7.5 (total volume 0.2 mL). The kinetics of the formation of spin adduct DMPO–$^{\bullet}CH_3$ were measured by the changes of a low-field sextet component of the EPR signal of DMPO–$^{\bullet}CH_3$ (Sushkov et al., 1987).

Electrochemical Measurements. The cyclic voltammetry experiments were performed with an LP-7E polarograph (Czechoslovakia) in nonaqueous DMSO in the presence of

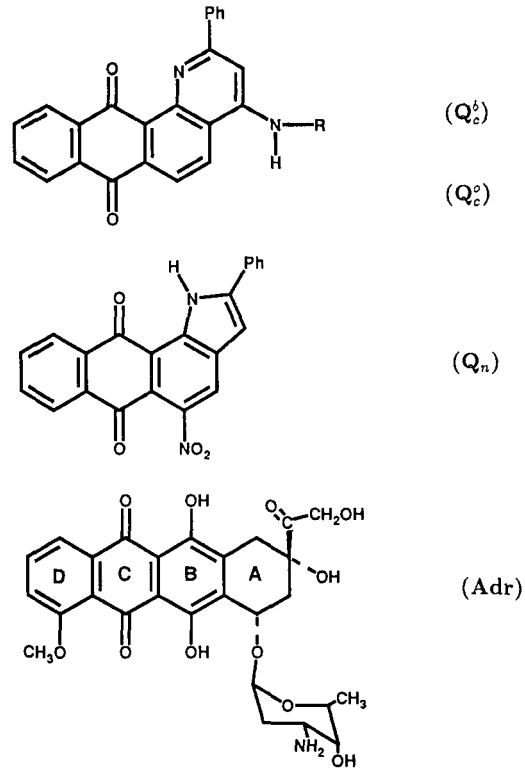


FIGURE 1: The chemical structures of quinones used: 2-phenyl-4-(butylamino)naphtho[2,3-*h*]quinoline-7,12-dione (Q_c^b; R = butyl); 2-phenyl-4-(octylamino)naphtho[2,3-*h*]quinoline-7,12-dione (Q_c^o; R = octyl); 2-phenyl-5-nitronaphtho[2,3-*g*]indole-6,11-dione (Q_n); adriamycin (Adr).

0.1 M tetraethylammonium perchlorate an an electrolyte. Reduction potentials were measured by tetraethylammonium perchlorate as an electrolyte. Reduction potentials were measured by a platinum electrode. The scanning rate was 5 V/min; the saturated calomel electrode served as reference.

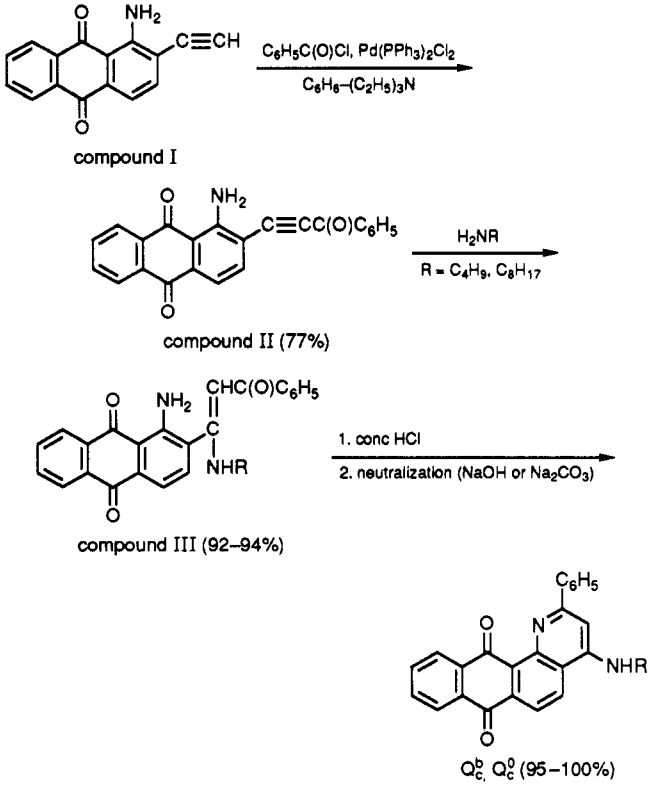
For measurements 1 mM solutions of quinones in DMSO were used. The concentration of $FeCl_3$ in solution was 0.5 mM. Prior to measurements, the sample was argon-bubbled for 10 min. First the potentials of quinones and $FeCl_3$ were independently measured, and then the potential of quinones was measured in the presence of iron ions.

Other Measurements. The EPR spectra were measured on E-12 (Varian) spectrometer in a flat sealed cell (volume 200 μL) at room temperature (22 °C). The experimental conditions were as follows: sweep width 100 G; microwave power 20 mW; modulation amplitude 0.8 G; receiver gain 2×10^4–10^5. NMR spectra were taken using AM-250 (Bruker) and FX-90Q (JEOL) spectrometers. Optical measurements were performed using a UVIKON 810 spectrophotometer.

Synthesis of the Quinones. 2-Phenyl-5-nitronaphtho[2,3-*g*]indole-6,11-dione (Q_n) (see Figure 1 for structures) was synthesized and characterized as described before (Shwartsberg et al., 1987). 2-Phenyl-4-(alkylamino)naphtho[2,3-*h*]-quinoline-7,12-diones (Q_c^b and Q_c^o) were synthesized according to Scheme I. The synthesis of the initial 1-amino-2-ethynylanthraquinone (compound I) is described (Piskunov et al., 1987). 1-Amino-2-(benzoylethynyl)anthraquinone (compound II) was synthesized using published procedures (Shwartzberg et al., 1990).

Generation of 1-Amino-2-[1-(butylamino)-2-benzoylethenyl]anthraquinone (Compound III). A solution containing 0.70 g of compound II and 2 mL of butylamine in 15 mL of dry benzene was stirred for 15 min under reflux, and the

Scheme I



solvent was removed under vacuum. A residue was stirred with a small volume of ether, and a solid was collected. The yield was 0.77 g (91%), mp 188.5–189.5 °C (benzene–ether). Anal. Calcd for $C_{27}H_{24}N_2O_3$: C, 76, 39; H, 5.70; N, 6.60. Found: C, 76.43; H, 5.80; N, 6.50.

1-Amino-2-[1-(octylamino)-2-benzoylethenyl]anthraquinone was obtained analogously. The yield was 92%, mp 123–124 °C (benzene–ether). Anal. Calcd for $C_{31}H_{32}N_2O_3$: C, 77, 47; H, 6.71; N, 5.83. Found: C, 77, 51; H, 6.80; N, 5.94.

Generation of 2-Phenyl-4-(butylamino)naphtho[2,3-h]-quinolinedione (Q_c^b). To a solution of 0.42 g of III in 200 mL of benzene was added 10 mL concentrated HCl, the mixture was shaken for 1 min, neutralized with a solution of Na_2CO_3 (or NaOH), and washed with H_2O, and the solvent was removed under vacuum. A residue was stirred with ether and hexane, and a solid was collected, yield 0.40 g (95%). 2-Phenyl-4-(octylamino)naphtho[2,3-*h*]quinolinedione (Q_c^b) was obtained analogously, yield 100%.

Characteristics of Synthesized Quinones. Characteristics of synthesized quinones Q_c^b and Q_c^o are presented in Table I.

RESULTS

Spectrophotometric Study of the Formation of Complexes of Quinone Q_c^b with Fe(III).[1] The formation of complexes of organic ligands with transition metal ions affects the ligand absorption spectrum, leading to disappearance and shfit of the bands and appearance of new bands (Martell & Calvin, 1952). The stability constant of a ligand–metal complex can be determined spectrophotometrically from the difference of the absorption spectra of ligands in a free state and bound in a complex.

We have examined the sensitivity of absorption spectra of quinones Q_c^b and Q_n to Fe(III) ions in solution. The measurements were made in chloroform solutions. As found, addition of $FeCl_3$ leads to the change only in the case of Q_c^b. Introduction of $FeCl_3$ resulted in both a 360-nm decrease and 500-nm increase in absorption (Figure 2). The isobestic points observed at titration of Q_c^b solution with increasing concentrations of $FeCl_3$ (Figure 2) indicate the presence of two individual compounds in solution.

The complex formation of the Q_c^b with Fe(III) was studied by the method of molar relations. From the analysis of saturation curves (Figure 3a) it follows that Q_c^b and Fe(III) form the complex whose maximum stoichiometry is 3:1.

In order to determine the stability constant of the FeQ_3 complex K, we found the difference between coefficients of molar extinction of Q_c^b in a free state in solution and in its complex with Fe(III) at 500 nm: $\Delta\epsilon = 3 \times 10^3\ M^{-1}\ cm^{-1}$. The dependence of Q_c^b absorption ($\lambda = 500$ nm) on the concentration of added $FeCl_3$ at a constant concentration of Q_c^b (C_0) was determined (Figure 3b).

The concentration of Q_c^b in the complex with iron is $C_c = \Delta A/\Delta\epsilon L$, where L is an optical pathway.

$$K = ([FeQ_3]/[Q]^3)[Fe] = C_c/(C_0 - 3C_c)^3(C_{Fe} - C_c)$$

The value of K was $(7 \pm 1) \times 10^{18}\ M^{-3}$. Note that for complex of Ga with α-pyridoanthraquinone in benzene the stability constant K is equal to $1.7 \times 10^5\ M^{-1}$ (stoichiometry 1:1) (Zaitsev et al., 1979). For comparison of the stability constant obtained by us for $Fe(Q_c^b)_3$ with that for GaQ assume that $K_1 \approx K_2 \approx K_3$, where $K_1 = [FeQ]/[Fe][Q]$, $K_2 = [FeQ_2]/[FeQ][Q]$, and $K_3 = [FeQ_3]/[FeQ_2][Q]$. Then $K = K_1K_2K_3 = K_i^3$; $K_i = 1.9 \times 10^6\ M^{-1}$.

Polarographic Study of Synthesized Quinones and Adriamycin. One-electron reduction potentials of quinones Q_b^c and Q_n and adriamycin in the presence and absence of Fe(III) are shown in Table II. It is seen that the one-electron reduction potentials of Q_n and adriamycin are similar and more positive than that of Q_c^b. Obviously, the presence of a nitro group in Q_n makes it a stronger oxidant than Q_b^c. In the presence of iron ions a shift of the Q_c^b and adriamycin potentials toward the positive region was observed, which seemed to reflect their ability to form a complex with Fe(III), as distinct from Q_n.

Thus, the experiments confirm the data on the formation of the Q_c^b complex with Fe(III) ions obtained from absorption spectra. The absolute values of one-electron reduction potentials of Q_c^b and Q_n similar to the values for adriamycin allow us to hope that these compounds will be reduced with NADPH–cytochrome P-450 reductase and stimulate the generation of OH radicals in the same way as anthracycline antibiotics according to reactions 1–5.

NADPH-Dependent Oxygen Radical Generation by Quinones Q_c^b and Q_n in the Enzymatic System. We used the capability of NADPH–cytochrome P-450 reductase to reduce the quinones that had antitumor properties and synthetic quinones (Bachur et al., 1978; Komiyama et al., 1982; Sushkov et al., 1987; Powis, 1989) to examine the capability of synthesized quinones to generate oxygen radicals via reactions 1–5.

Quinones Q_n and Q_c^b stimulated the oxidation of NADPH by NADPH–cytochrome P-450 reductase. For Q_n the rate of NADPH oxidation was 39 μM/min; for Q_c^b, 21 μM/min. The addition of iron chloride only slightly affected the rate of NADPH oxidation.

Kinetic of DMPO–$^{\bullet}CH_3$ adduct formation by Q_c^b, Q_n and adriamycin are shown in Figure 4. (Note that in the absence of quinones spin adduct formation was not detected.) It was

[1] Under identical experimental conditions quinone Q_c^o revealed the same properties as quinone Q_c^b.

Table I: Elementary Analysis and PMR Data of Synthesized Quinones

Q	mp, °C (solvent)	found/calcd, % C	H	N	PMR spectrum ($CDCl_3$), δ, ppm
Q_c^b (I)	>160 dec (C_6H_6–ether)	79.64/79.78	5.49/5.46	6.96/6.89	1.00 (t, 3 H, CH_3), 1.20–1.75 (m, 4 H, CH_2CH_2), 3.25–3.32 (m, 2 H, NCH_2), 5.10 (br s, 1 H, NH), 6.89 (s, 1 H, H^3), 7.47–7.59 (m, 3 H, Ph), 7.70–7.80 (m, 2 H, $H^{9,10}$), 8.08–8.33 (m, 6 H, $H^{5,6,8,11}$, Ph)
Q_c^o (II)	>140 dec (C_6H_6–hexane)	80.25/80.49	6.57/6.54	6.17/6.06	0.89 (t, 3 H, CH_3), 1.24–1.85 [m, 12 H, $(CH_2)_6$], 3.4 (m, 2 H, NCH_2), 5.10 (br s, 1 H, NH), 6.95 (s, 1 H, H^3), 7.48–7.56 (m, 3 H, Ph), 7.72–7.80 (m, 2 H, $H^{9,10}$), 8.17–8.38 (m, 6 H, $H^{5,6,8,11}$, Ph)

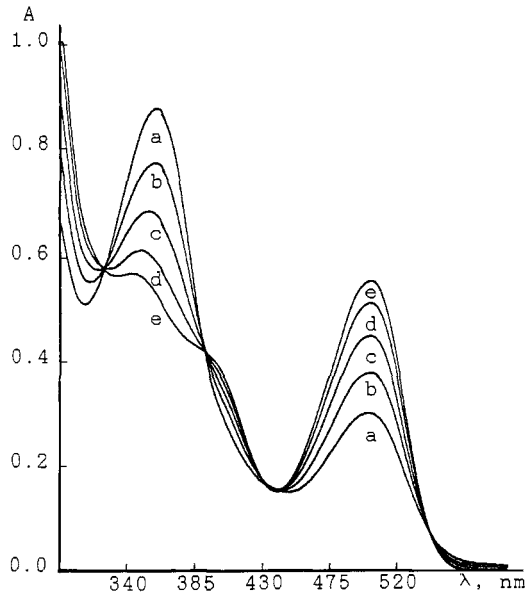


FIGURE 2: Absorption spectra of Q_c^b in the absence and presence of $FeCl_3$. Concentration of Q_c^b = 50 μM. Solvent:chloroform. Concentrations of $FeCl_3$: (a) 0.0; (b) 4.5 μM; (c) 9 μM; (d) 13.5 μM; (e) 18 μM.

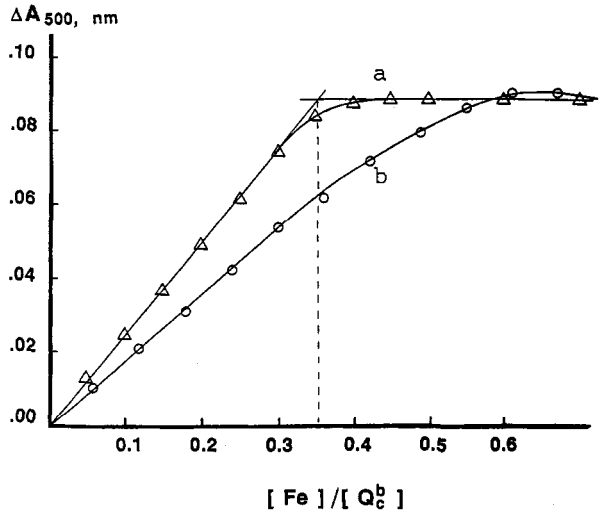


FIGURE 3: Dependence of optical absorption of Q_c^b at λ = 500 nm on $FeCl_3$ concentrations. Solvent:chloroform. (a) Concentration of Q_c^b = 10 μM. Optical path, L = 1 cm. (b) Concentration of Q_c^b = 3.33 μM, L = 3 cm.

shown earlier for synthetic quinones and antitumor antibiotics (in a hermetically sealed EPR flat cell) that the kinetics of spin adduct formation in enzymatic redox system consisted of the following main stages: lag period, rapid formation of spin adduct, plateau, and slow decay of EPR signal of spin adduct (Sushkov et al., 1987; Rumyantseva & Weiner, 1988). As the decay reflects complex processes of reduction and decay of the nitroxyl radical formed, for the qualitative evaluation

Table II: One-Electron Reduction Potentials of Quinones in Nonwater Dimethyl Sulfoxide (V)[a]

quinone	adriamycin	Q_n	Q_c^b
$-FeCl_3$	−0.55	−0.53	−0.70
$+FeCl_3$	−0.45	−0.53	−0.54

[a] The concentrations of quinones and $FeCl_3$ and experimental conditions were described in Materials and Methods.

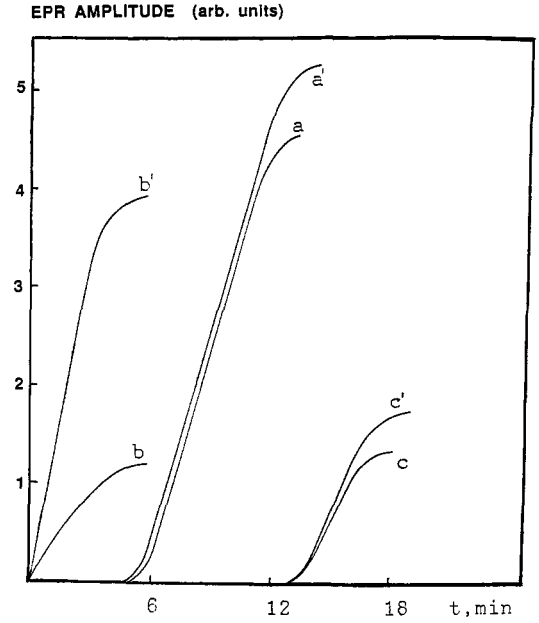


FIGURE 4: Kinetics of the formation of DMPO–•CH_3 spin adduct in the enzymatic system containing (a) 50 μM adriamycin, (b) 50 μM quinone Q_c^b, or (c) 50 μM quinone Q_n. (a′–c′) Kinetics after addition of $FeCl_3$ (25 μM). Reaction mixture and experimental conditions for EPR measurements are described in Materials and Methods.

of the efficiency of OH radical formation by the quinones of interest here, one can compare values of lag periods, rates of spin adduct formation, and maximum amplitudes of EPR spectra. As can be seen in Figure 4, all the quinones can generate hydroxyl radicals. However, unlike other quinones, OH radicals in the samples with Q_c^b occur without a lag period. Also, in the presence of $FeCl_3$ the largest increase of the amplitude of the spin adduct EPR signal was also observed in the sample with quinone chelator Q_c^b (Figure 4).

To study the role of iron ions in OH radical formation for Q_c^b, we have studied the kinetics of OH radical formation depending on the concentration of added $FeCl_3$ (Figure 5). It was found that the addition of $FeCl_3$ to the samples with Q_c^b leads to the growth of both the initial effective rate of spin adduct formation and the maximum amplitude of EPR signal of spin adduct.

In order to determine the influence of the interaction of Q_c^b and Q_n with Fe(III) ions on OH radical production, we have plotted the dependence of the maximum amplitude of

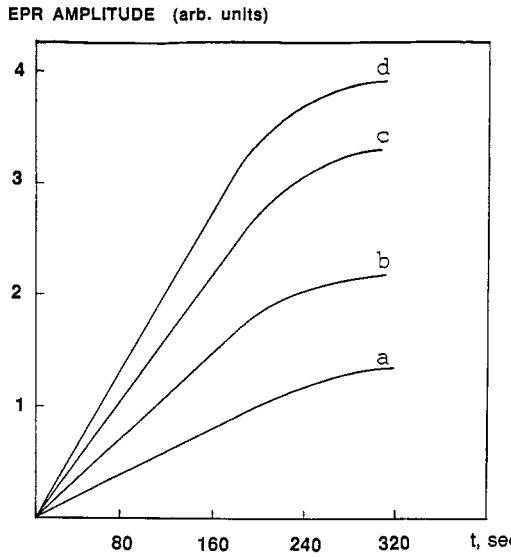


FIGURE 5: Kinetics of the formation of DMPO–$^{\bullet}CH_3$ spin adduct in the enzymatic system with quinone Q_c^b (50 μM) after addition of $FeCl_3$. Concentrations of $FeCl_3$: (a) 0.0; (b) 6 μM; (c) 12 μM; (d) 25 μM.

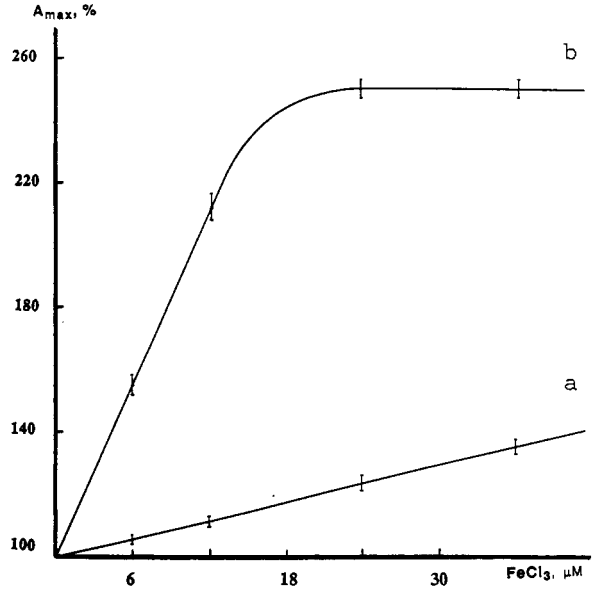


FIGURE 6: Dependences of maximum amplitude (A_{max}) of the EPR signal of the DMPO–$^{\bullet}CH_3$ spin adduct (see Figure 5) on the concentration of the $FeCl_3$ in enzymatic system. (a) 50 μM quinone Q_n; (b) 50 μM quinone Q_c^b.

the spin adduct EPR signal on the concentration of added Fe(III) (Figure 6). As is seen in Figure 6, these dependences are dramatically different for Q_c^b and Q_n: for Q_n the amplitude of the spin adduct linearly increases with increasing Fe(III) concentration in solution. For Q_c^b this dependence has a much stronger slope (tangents of inclination angles differ by almost a factor of 6), and for the $[Q_b^c]/[Fe^{3+}]$ ratio ~2 it reaches the plateau.

DISCUSSION

As follows from the structural formula of the quinone obtained, Q_c^b (Figure 1), it has atoms capable of forming the coordination bond with metal ions. These atoms are the oxygen atom of the carbonyl group and the nitrogen atom of the pyridine ring; in other words, the quinone Q_c^b is a bidentate ligand and, in principle, can form chelate complexes with Fe(III) ions. The nitrogen atom in the pyrrole ring of the quinone Q_n is a weak metal ligand; therefore, this quinone with a structure similar to that of Q_c^b was used by us as a possible nonchelating control.

Our experiments have shown that Q_c^b, in fact, can form complexes with Fe(III). This conclusion follows from the changes in absorption spectrum of Q_c^b caused by Fe(III) ions (Figure 2). Note that introduction of Fe(III) ions to the solution containing quinone Q_n did not lead to any changes in absorption spectra. The stability constant, determined by the molar relations method, was found to be similar to that of Ga ions bound to α-pyridoanthraquinone (Zaitsev et al., 1979).

It should be noted that Fe(III) ions cause changes in absorption spectra of adriamycin (Eliot et al., 1984; Beraldo et al., 1985). It was supposed that coordinating atoms in the $Fe(Adr)_3$ complex are oxygen C-11 and oxygen C-12 of adriamycin (Eliot et al., 1984). Proceeding from the structure and donor properties of Q_c^b, by analogy with the α-pyridoanthraquinone complex with Ga (Zaitsev et al., 1979), one can suppose that the coordinating ligands in the Q_c^b – Fe (III) complex are also the oxygen of the carbonyl group and the nitrogen of the pyridine ring. If Fe(III) can coordinate to these groups, a six-member stable cycle will arise.

The shift of one-electron reduction potentials of Q_c^b and adriamycin toward the positive region in the presence of Fe(III) ions and the absence of an influence of Fe(III) on the Q_n one-electron reduction potential (Table II) are evidence in favor of formation of Fe(III) complexes with Q_c^b and adriamycin. Since when proceeding to more polar solvents, the absolute values of one-electron reduction potentials of quinones can be more positive (Rumyantseva et al., 1986), one can suppose that in the buffer solution the Q_c^b – Fe (III) complex will be an efficient electron acceptor in biological electron-transfer chains, i.e., will stimulate reactions 1–5.

As is seen in Figure 4, all the quinones studied stimulate the formation of OH radicals in the enzymatic system. We measured two parameters characterizing the process: the absolute amplitude of the EPR signal of the spin adduct $^{\bullet}CH_3$–DMPO and duration of the lag period (Sushkov et al., 1987; Rumyantseva & Weiner, 1987; Dikalov et al., 1991). It should be noted that quinone Q_c^b stimulated the formation of OH radicals without any lag period and addition of Fe(III) ions significantly increased the rate of OH radical generation and concentration of the spin adduct in the case of Q_c^b (Figures 5 and 6).

We believe that the mechanism of OH radical generation in the course of redox transformations of quinones (eqs 1–5) is also valid in the absence of artificially added Fe(III). The mechanism can be explained by the presence of submicromolar amounts of transition metal ions, which are always found in buffer solutions (Morehouse & Mason, 1988). For the systems in question this is evidenced by stimulation of OH radical generation by $Fe(EDTA)_2$ (Sushkov et al., 1987; Rumyantseva & Weiner, 1988) and its inhibition by desferrioxamine, a chelator whose complex with Fe ions is redox-inactive (Kalynaraman et al., 1991).

The different dependences of the maximal amplitude of the EPR signal of the spin adduct on concentration of Fe(III) for the chelating quinone (Figure 6) can be due to the formation of the Q_c^b–Fe(III) complex. The stoichiometry of this complex in water was 2:1, as follows from Figure 6b. The question arises: why do the absorption spectra of Q_c^b (Figure 3) suggest that the stoichiometry of the Q_c^b – Fe(III) complex is 3:1, while saturation (from the measurements of the spin adduct)

is observed at Q_b^c/Fe(III) ~2:1 (Figure 6b)? Note that our optical data are for complex of Q_c^b with Fe(III) in chloroform solution, while experiments on OH radical generation were performed in water buffer, where the Q_c^b/Fe(III) stoichiometry might differ from that in chloroform solution.[2] It is not excluded either that $\epsilon_{500nm}(FeQ_3) = \epsilon_{500nm}(FeQ_2)$ in chloroform for quinone Q_b^c. This precludes the possibility of measuring constants of formation of possible Q_c^b – Fe(III) complexes with the stoichiometries 1:1 and 2:1.

It is known that in the presence of quinones the rate of NADPH-dependent reduction of quinones by NADPH–cytochrome P-450 reductase depends on the one-electron reduction potential of the quinone. The more electropositive quinones were more rapidly reduced than the less electropositive ones (Powis & Appel, 1981; Cenas et al., 1984). As follows from Table II, the one-electron reduction potentials of Q_b^c and Q_n in the presence of Fe(III) ions are similar. For this reason, the differences in dependences of the OH radical generation rate and of the maximum amplitude of EPR signal of spin adduct on concentration of Fe(III) for these quinones (Figures 5 and 6) cannot be explained by the difference in quinone one-electron reduction potentials.

During the course of Q_c^b – FeIII complex formation we observed a shift of its redox potential by 0.16 V toward the positive region (Table II). However, we did not detect any noticeable effect of this complex on the NADPH oxidation rate, which was predictable from thermodynamic considerations. One can suppose that the electron-transfer rate from the reduced NADPH–cytochrome P-450 reductase into quinone is determined not only by redox potentials of enzyme and quinone but also by accessibility of the enzyme active center to interaction with the electron acceptor (steric factor). One can assume that the reductase active center is less accessible for a complex of Q_c^b with Fe(III) than that of Q_c^b alone.

What does the absence of the lag period in OH radical formation observed for Q_c^b mean? The mechanism of the lag period formation before the appearance of OH radical spin adduct in described redox quinone-containing systems was explained in terms of the reactions 1–5 (Sushkov et al., 1987; Rumyantseva & Weiner, 1988). In particular, it was shown that the duration of the lag period is directly proportional to oxygen concentration in the solution. Hence, the lag period was assumed to equal the time of oxygen reduction and the hydrogen peroxide production via reactions 1–3. Then, the iron ions are reduced according to reaction 4, and reaction 5 (with OH radical formation) starts. This mechanism can be used to account for the presence of a lag period for Q_n and for its absence for Q_c^b. Since the rate constant of reaction 2 is usually equal to or higher than the rate constant of reaction 4 [for example, for reduction of molecular oxygen by semiquinone of adriamycin, $k_2 = 3 \times 10^8$ M^{-1} s^{-1}, and for reduction of Fe(III)–EDTA complex by semiquinone of adriamycin, $k_4 = 2.8 \times 10^8$ M^{-1} s^{-1} (Butler et al., 1985)], for quinone Q_n as for other known quinones (Sushkov et al., 1987; Rumyantseva & Weiner, 1988; Kalynaraman et al., 1991) the presence of the lag period can be caused by the competition between reactions 2 and 4.

We think that in the case of Q_c^b the rate ratio of these reactions is likely to change due to reduction of Fe(III) in the complex with semiquinone of Q_c^b, which leads to Fe(II) formation. An increase in the efficient of OH radical generation and higher values of the concentration of spin adduct after addition of Fe(III) to the system (Figures 5 and 6) is, probably, determined by an increase in concentration of the complex of Q_c^b with Fe(III) ions.

Thus, the proposed intramolecular reduction of Fe(III) in the complex with the quinone (semiquinone) Q_c^b can lead to the formation of Fe(II) ions in the presence of oxygen, i.e., to the initiation of reaction 5. In this case the formation of OH radicals can occur without a lag period.

For OH radical generation via reactions 1–5 the existence of a semiquinone anion–Fe(III) complex is essential. It can be supposed that the semiquinone anion of Q_c^b can also form complexes with Fe(III). It is known that Fe(III) can form quite stable complexes with semiquinones of substituted benzoquinones (Buchan et al., 1978; Lynch et al., 1982; Boone et al., 1989). In this case an intramolecular electron transfer occurs in solutions with a high dielectric constant (Lynch et al., 1982).

The dependence of the maximal EPR signal amplitude of spin adduct for Q_n on concentration of Fe(III) is a straight line with a constant inclination tangent (Figure 6a). This fact may be due to a linear dependence of reaction rate 5 on concentration of Fe(II) ions.

The question naturally arises: why, for adriamycin, which also can form stable complexes with Fe(III) ions (Zweier, 1984; Beraldo et al., 1985), is a marked lag period in the kinetics of spin adduct formation in the presence of Fe(III) ions nevertheless observed? It should be said first of all that the elucidation of the mechanism of Fe(III) ion reduction in the complex with adriamycin was not the objective of this work. This problem has been studied in detail by some researchers [see review of Powis (1989) and references therein] but still remains unsolved. Particularly, note a nontrivial fact of reduction of Fe(III) in the complex with adriamycin in the absence of electron donors (Zweier, 1984; Gianni et al., 1985). This observation probably reflects the dual nature of adriamycin: it is an electron acceptor (quinone structure, ring C) and simultaneously an electron donor (hydroquinone structure, ring B) (Figure 1). In addition, it should be taken into account that hydrogen bond formation between the OH group in position 6 and the carbonyl oxygen 5 in the adriamycin molecule can provide stabilization of the semiquinone radical. This fact has been convincingly proven for semiquinones of numerous substituted naphtho- and anthraquinones (Ashnager et al., 1984). In principle, this event can impede the intramolecular electron transfer from semiquinone into Fe(III) ion. These two ideas, which of course are not the only ones, indicate the complex redox behavior of Adr–Fe(III) complexes.

CONCLUSION

Thus the main result of this work is experimental confirmation of the hypothesis that quinones capable of forming complexes with Fe(III) ions stimulate the formation of oxygen radicals in biological redox systems more efficiently than their nonchelating analogues. We propose that this may be due to intramolecular electron transfer from the semiquinone into Fe(III) ion. This finding is important both for the purpose of development of new physiologically active quinone-containing compounds [so-called bioreductive drugs (Richards & Reynolds, 1990)] and for the synthesis of quinones covalently bound to oligonucleotides which are complementary

[2] Addition of Fe(III) to a buffer solution of Q_c^b did not affect the optical spectra of the quinone. For this reason it is impossible to independently measure the complex formation in water.

to a definite site of nucleic acid for site-specific nucleic acid scission (Mori et al., 1989; Dikalov et al., 1991).

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