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SUPEROXIDE RADICAL REACTIONS WITH ANTHRACYCLINE ANTIBIOTICS

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Abstract—The reaction of superoxide with daunorubicin or its aglycones in the aprotic solvents dimethyl sulfoxide and dimethylformamide was studied. This interaction generated the blue anthracycline phenolate anion as monitored by u.v.-visible spectrometry and molecular oxygen as determined by a modified Clark-type oxygen electrode. The visible spectrum of the phenolate anion (λ_{max} 604, 652 nm) was subject to considerable shifts dependent on the size of the cation present. The phenolate anion could be further oxidized by molecular oxygen to generate the C-6, C-11 (B-ring) semiquinone as detected by a weak electron paramagnetic resonance spectrometry signal. These results raise the possibility that similar reactions of superoxide with anthracyclines *in vivo* may play a role in the antitumor activity and/or the etiology of the toxic side effects of this class of drugs.

In our studies of anthracycline antibiotic pharmacology, we have shown that quinone containing anticancer agents are enzymatically activated to free radical forms by reduced flavoproteins such as NADPH cytochrome P-450 reductase [1–3], xanthine oxidase [4] and NADH cytochrome *c* reductase [5]. These free radicals can then transfer a single electron to molecular oxygen to form superoxide and the regenerated antibiotic [1, 6–8]. This interaction of cellular enzymatic reductive potential, the quinone antibiotic, and oxygen is proposed to be central to the cytotoxicity of this class of antibiotics, especially in explaining their cytodestructive characteristics [2, 9, 10].

Besides being generated secondarily from the anthracycline free radical, superoxide anion, O_2^- is also a common respiratory intermediate of aerobic organisms and is formed through many biochemical reactions [11–13]. Although superoxide concentration is controlled intracellularly by superoxide dismutase, the introduction of xenobiotics, such as anthracyclines, into biological systems may result in net reactions with these controlled levels of superoxide to generate cytotoxic products or intermediates [14]. The reactivity of superoxide with anthracyclines is thus of particular interest, not only from the viewpoint of the pharmacological activity of these drugs but also for toxicologic considerations.

Recently, interest has focused on the interactions of anthracyclines with cell membranes. Changes in the surface membranes of Sarcoma 180 ascites cells were induced by doxorubicin as measured by an increase in the rate of agglutination of cells of concanavalin A [15, 16] and by an increase in membrane fluidity as measured by electron paramagnetic resonance spectrometry [16]. In the mitochondrial membrane, doxorubicin and derivatives were found to inhibit the last oxidation site of the respiratory chain (cytochrome *c* oxidase) by excluding the enzyme from its essential cardiolipin environment [17]. Doxorubicin affects plasma membrane redox function by stimulation of NADH oxidase and inhibition of ascorbate oxidase [18]. Plasma membrane changes have been identified as factors in the development of resistance to daunorubicin [19, 20]. In addition, if doxorubicin is covalently bound to solid polymeric supports, it is theoretically limited to cell-surface interactions. Doxorubicin thus coupled to polyglutaraldehyde microspheres retains its full activity against sensitive cell lines and has increased lethality to resistant cell lines [21, 22]. Tritton and Yee [23] have also shown that doxorubicin covalently bound to agarose beads is not released, does not enter the cells, and yet is cytotoxic to L1210 cells in culture. The cytotoxicity in these studies was apparently mediated solely by a cell-surface interaction.

The anthracyclines have considerable solubility in polar organic solvents as reflected by a 1-octanol:-Tris-buffer partition coefficient of 0.52 [24]. The aglycones, which have lost the amino sugar daunosamine, are soluble in polar and nonpolar organic solvents. These antibiotics can thus be expected to attain reasonable concentrations in phospholipid bilayers constituting living membranes, particularly if one reasons that the hydrophobic tetracyclic portion of the molecule is inserted into the nonpolar interior of the membrane while the polar daunosamine moiety is held at the surface by the negatively charged phosphate groups. The study of the basic chemistry of anthracyclines in polar organic

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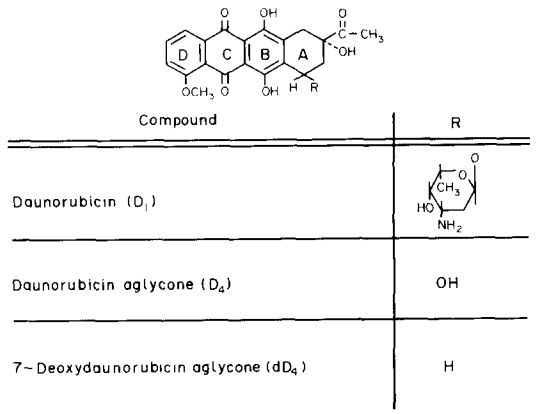


Fig. 1. Structures of daunorubicin (D_1), daunorubicin aglycone (D_4), and 7-deoxydaunorubicin aglycone (dD_4).

solvents may thus have important ramifications in improving our understanding of interactions of anthracyclines with cellular membranes and with other hydrophobic regions of cell such as the interior of the DNA double helix or hydrophobic pockets in enzymes.

To understand better the possible intracellular interactions of superoxide with anthracyclines, we have investigated the reactions of superoxide with daunorubicin (D_1), daunorubicin aglycone (D_4), 7-deoxydaunorubicin aglycone (dD_4) (Fig. 1), and the model compound quinizarin in polar aprotic solvents, an environment that may mimic that of anthracyclines dissolved in a biological membrane. Although a previous report has addressed this interaction [25], that study incorrectly concluded that the principal reaction was electron transfer from superoxide to doxorubicin. We have used both absorption spectrophotometry and electron paramagnetic resonance spectrometry to demonstrate that the principal reaction of either crown ether solubilized potassium superoxide, or electrochemically generated superoxide with these compounds in the aprotic solvents dimethyl sulfoxide and dimethylformamide was the abstraction of a phenolic proton to generate the phenolate anion.

MATERIALS AND METHODS

Daunorubicin hydrochloride (D_1) was obtained from the Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD), and was purified by silicic acid column chromatography [26]. Daunorubicin aglycone (D_4) was prepared by acid hydrolysis of D_1 and purified as previously described [27]. The 7-deoxydaunorubicin aglycone (dD_4) was prepared by reductive cleavage of D_1 with sodium dithionite [28]. Silicic acid column chromatography was used for partial purification of dD_4. For further purification of dD_4, countercurrent chromatography was employed [29]. Dimethyl sulfoxide (DMSO), spectrophotometric grade, and dicyclohexano-18-crown-6 were obtained from the Aldrich Chemical Co. (Milwaukee, WI). Dimethylformamide (DMF) was from Burdick & Jackson Laboratories (Muskegon, MI). Potassium superoxide and 1,4-dihydroxy-9,10-anthraquinone (quinizarin) were obtained from ICN Pharmaceutical, Inc. (Plainview, NY). Tetrabutylammonium percholate (Alfa Products, Danver, MA) and tetraethylammonium perchlorate (TEAP) (Kodak Co., Rochester, NY) were used without further purification. Tetrabutylammonium hydroxide (40% in water) was obtained from the Sigma Chemical Co. (St. Louis, MO).

Superoxide ion was conveniently generated in polar aprotic solvents by either of two methods. The first used potassium superoxide solubilized in DMSO with the crown ether, dicylohexano-18-crown-6 [30–32]. A second method produced superoxide by electrochemical reduction of aerated DMF at −1.000 V vs Ag/AgCl, 3 M NaCl [25, 32, 33] with an electrochemical flow cell constructed as previously described [34]. The electrolyte was 0.1 M TEAP.

Absorption spectral studies. Ultraviolet-visible absorption spectra were obtained on an Aminco DW-2 UV-Visible spectrophotometer (Silver Spring, MD) in a 1.0 cm light path quartz cuvette. Repetitive spectral scans were obtained automatically at 10 nm/sec after addition of a KO_2 crown ether complex in DMSO to a solution of anthracycline in DMSO.

Measurement of oxygen production. Oxygen production was determined with a Clark-type electrode in a model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co.). To measure the oxygen in aprotic solvents, we modified a Clark-type electrode by making the outside jacket of Teflon and replacing saturated KCl with 2.0 M tetrabutylammonium perchlorate in DMSO. The test solution was bubbled with nitrogen for 3 min, and the electrode was placed in contact with the solution. After 1-min equilibration, 0.01 M anthracycline drug solution was injected into the reaction mix. After the base line was stabilized for 1 min, the KO_2 crown ether complex in DMSO was mixed into the reaction vessel, and oxygen concentration was monitored and recorded on a 7132A Hewlett–Packard recorder (Avondale, PA). All measurements were made at room temperature.

Electron paramagnetic resonance (EPR) spectrometry. EPR spectra were obtained with a Varian X-band E-109 EPR spectrometer fitted with a dual rectangular (TM-104) cavity. The field modulation frequency was 100 kHz. A strong pitch standard ($g = 2.0028$) was used in the reference cavity to measure g values. Scan conditions are stated in the figure legends.

Thin-layer chromatography (TLC). The identity and purity of the anthracycline compounds were assessed by TLC on silica gel 60 and silica gel F-254 plates (E. Merck, Darmstadt, West Germany). The solvent systems were either chloroform–methanol–acetic acid–water (80:20:14:6) or chloroform–methanol–acetic acid (100:2:2.5).

RESULTS

The reactions of D_1, D_4, and dD_4 dissolved in DMSO or DMF with superoxide were characterized by the change of light absorption maxima in the range 300–750 nm (Fig. 2). Immediately after the addition of the KO_2 crown ether complex to the anthracyclines in DMSO, a rapid decrease in absorb-

ance intensity at 480 and 505 nm and the appearance of a new absorbance with maxima at 604 and 652 nm occurred. These spectral changes were accompanied by the change in solution color from red-orange to blue. Repetitive spectral scans at room temperature to 1.0 hr showed that the new absorbance at 604–652 nm disappeared, and the blue color faded for D_1 and dD_4 (Fig. 2). Identical spectral changes were observed when electrochemically generated superoxide was used and reactions were carried out in DMF.

After obtaining light spectra of D_1, D_4, and dD_4

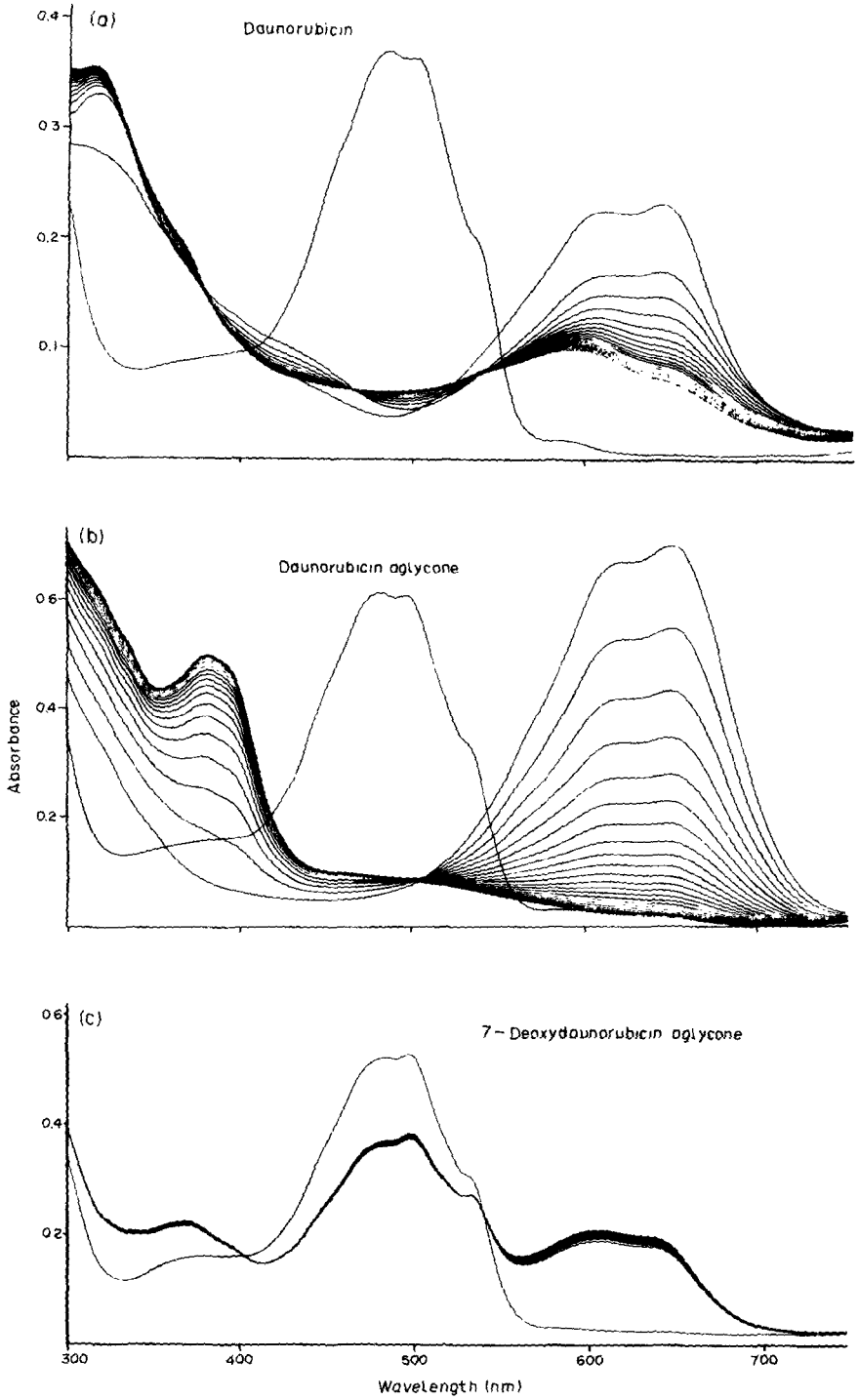


Fig. 2. Absorption of D_1, D_4 and dD_4 mixed with KO_2 crown ether complex in DMSO. The reaction mixture contained approximately 5×10^{-5} M drug, and a 10-fold excess of KO_2 crown ether complex in DMSO.

(spectra a of Fig. 3), the KO_2 crown ether complex was added, and the blue-colored products were immediately extracted by the addition of chloroform and water. The color of the chloroform extracts changed from blue to the original red-orange color for each compound, and absorption spectra indicated this visible change (spectra b of Fig. 3). TLC of the chloroform extracts verified the unchanged nature

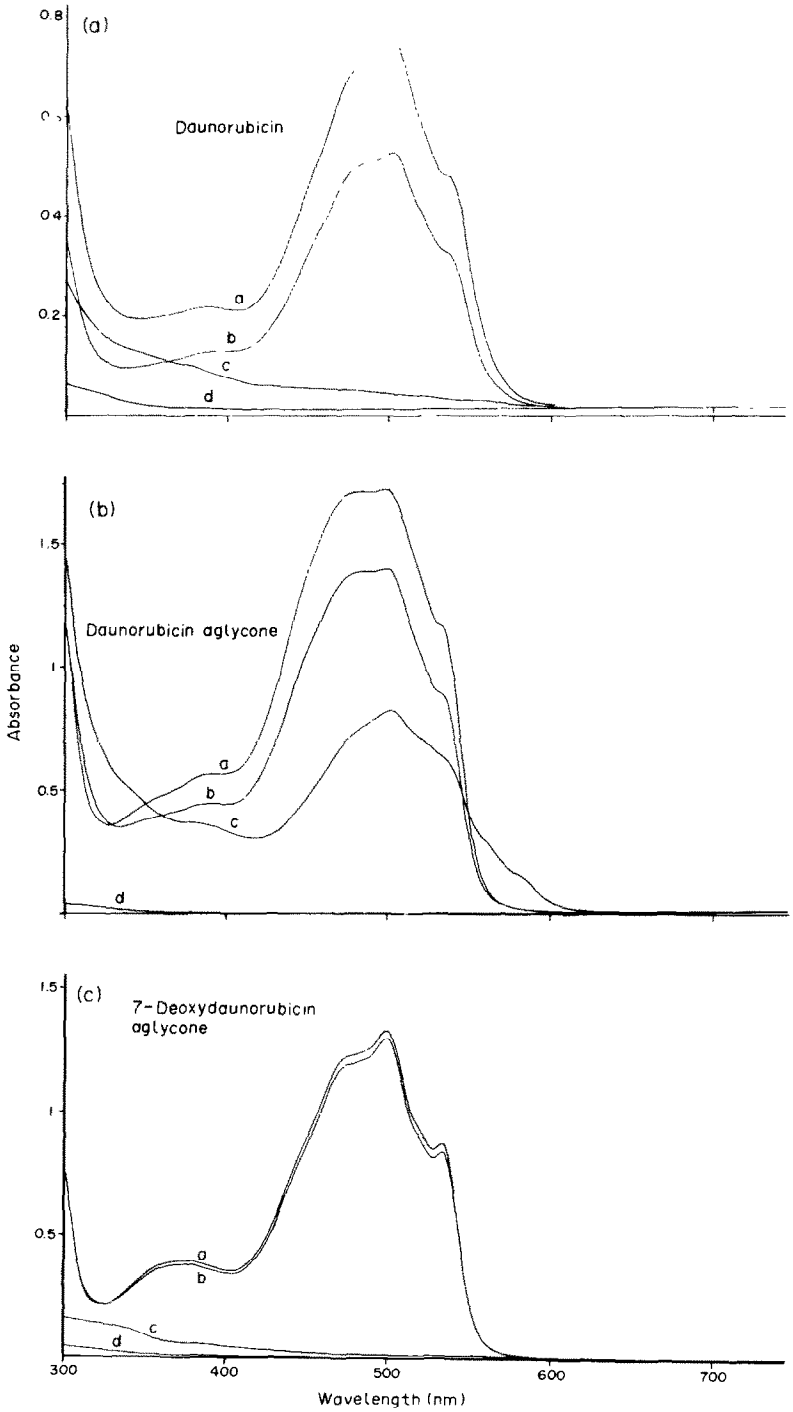


Fig. 3. Comparison of absorption spectra of D_1, D_4 and dD_4 before and after the addition of KO_2, and extraction with water/chloroform. Key: (a) Spectrum of anthracycline drugs in DMSO. (b) Spectrum after the reaction of KO_2 with anthracycline drugs and the addition of 1.0 ml H_2= and 2.5 ml chloroform. The spectrum of the chloroform layer was taken. (c) Spectrum after KO_2 reacted with anthracycline for 1.5 hr and then extracting with 1.0 ml water and 2.5 ml chloroform. The spectrum of the chloroform layer was taken. (d) Spectrum of crown ether solubilized KO_2 in DMSO at the concentration used for reaction with anthracyclines.

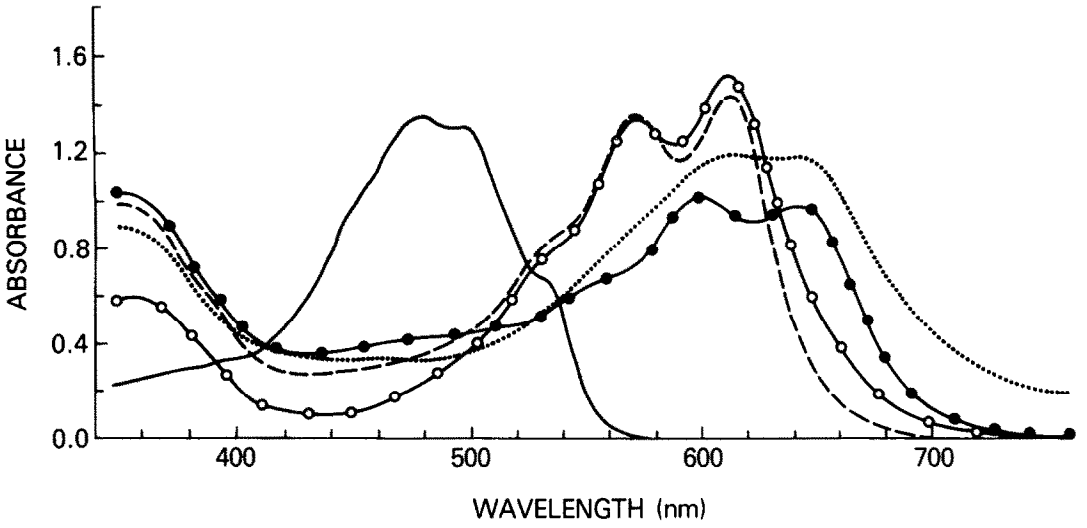


Fig. 4. Absorbance spectra of D_1 with various bases; D_1 (——), D_1 and superoxide (●—●—●), D_1 and NaOH (○—○—○), D_1 and superoxide plus NaCl (- - - -), and D_1 and tetrabutylammonium hydroxide (·····).

of the starting materials. Similarly, at 1.5 hr after the addition of the KO_2 crown ether complex to these anthracyclines, absorption spectra were obtained of the chloroform extract after extraction with chloroform and water (spectra c of Fig. 3). The spectra of D_1 and dD_4 reactions are nearly that of solvent and KO_2 crown ether complex alone (spectra d of Fig. 3), whereas D_4 has shown partial recovery of an anthracycline chromophore (Fig. 3b). TLC of these extracts showed complete degradation of D_1 and dD_4. No fluorescent or u.v.-absorbing products were observed.

Since it was known that D_1 forms a blue species under basic conditions due to ionization of a phenolic proton [35], we suspected that the blue species generated by superoxide was also a phenolate anion. To investigate this suspicion, we titrated D_1 in DMF (0.1 M TEAP) with 0.1 M NaOH. A blue solution resulted having light absorbance maxima at 572 and 611 nm (Fig. 4).

Since the NaOH generated spectrum had the same envelope as the superoxide generated species, but was shifted hypsochromically, we determined the effect of sodium ion on the absorbance spectrum. When a portion of 0.6 N sodium chloride was added to a solution of the blue species generated by reaction

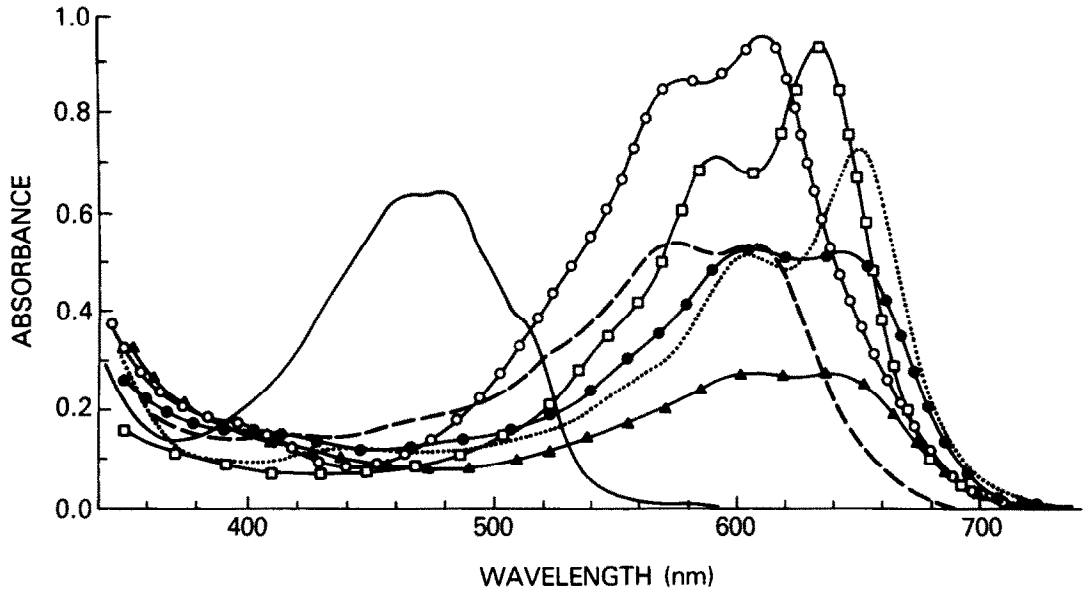


Fig. 5. Absorbance spectra of 1,4-dihydroxyanthraquinone (DHAQ) with various bases: DHAQ (——), DHAQ and superoxide (●—●), DHAQ and NaOH (○—○), DHAQ and superoxide plus NaCl (- - - -); DHAQ and tetrabutylammonium hydroxide (·····), DHAQ and KOH (□—□), and DHAQ and KOH plus crown ether (▲—▲). All spectra were recorded in DMF (0.1 M TEAP).

of D_1 with electrochemically generated superoxide, the absorbance spectrum shifted from the maxima at 602 and 643 nm to maxima at 570 and 611 nm and was identical to the spectrum obtained from NaOH treatment of D_1 (Fig. 4). Addition of an equivalent amount of water instead of the sodium chloride solution caused a slight shift to 596 and 635 nm, similar to the shift observed as this blue species degrades (Fig. 2). To confirm that the base-generated blue species of the anthracyclines was the same as the superoxide generated species and that the cation present was dramatically affecting the absorbance spectrum, we then added tetrabutylammonium hydroxide to a D_1 solution in DMF (0.1 M TEAP). As predicted, since the tetrabutylammonium cation is large relative to the sodium cation and similar to the tetraethylammonium cation present from the electrolyte, a blue color change occurred with a spectrum very similar to that produced with superoxide (Fig. 4).

To confirm these observations with the anthracycline compounds, we performed similar experiments with a simple model for the dihydroxyanthraquinone nucleus of the anthracyclines, quinizarin (1,4-dihydroxy-9,10-anthraquinone). The results from these studies (Fig. 5) corroborate the observations with the anthracyclines. The presence of alkali earth metal shifted the spectrum hypsochromically. The maxima of the superoxide generated spectrum and tetrabutylammonium hydroxide spectrum were virtually identical. When the crown ether was added to the solution after the spectrum had been shifted hypsochromically with KOH, the spectrum then shifted bathochromically to its original position, indicating that the potassium ions had been entrapped by the crown ether (Fig. 5).

These results led to the conclusion that the blue species obtained by reaction of anthracyclines with superoxide in aprotic solvents was the phenolate anion generated by abstraction of a phenolic proton (C-6 or C-11).

These same reaction mixtures were also studied by EPR spectrometry. The reaction of D_1, D_4, and dD_4 with KO_2 crown ether complex in DMSO generated a weak free radical signal without hyperfine structure (Fig. 6). D_1, D_4, dD_4 or the KO_2 crown ether complex alone in DMSO yielded no detectable free radical signals. After the addition of KO_2 crown ether complex to anthracycline drugs, the radical signals appeared rapidly with the blue color. The free radicals were measurable up to 1.0 hr. Identical EPR spectra were obtained by reaction with electrochemically generated superoxide in DMF (0.1 M TEAP). Calculated g values for the free radicals of D_1, D_4, and dD_4 in DMSO were 2.0056, 2.0056, and 2.0059 respectively. The g value for the free radical of D_1 in DMF was 2.0057 (Fig. 7a).

The treatment of doxorubicin with base has been reported to generate a weak EPR signal [36]. When we treated D_1 in DMF (0.1 M TEAP) with NaOH, the blue color change described above occurred, and a very weak and broad EPR signal was detected, g = 2.0052 (Fig. 7d). We next treated the superoxide generated free radical with NaCl and observed a broadening of the signal and shift in the g value to 2.0053 (Fig. 7b). An equivalent amount of water (10 μl) had no effect on the spectrum (Fig. 7c). These shifts were completely analogous to those observed in absorbance spectrophotometry in which the superoxide generated spectrum and NaOH generated spectrum could be interconverted by simple addition or removal of sodium ion.

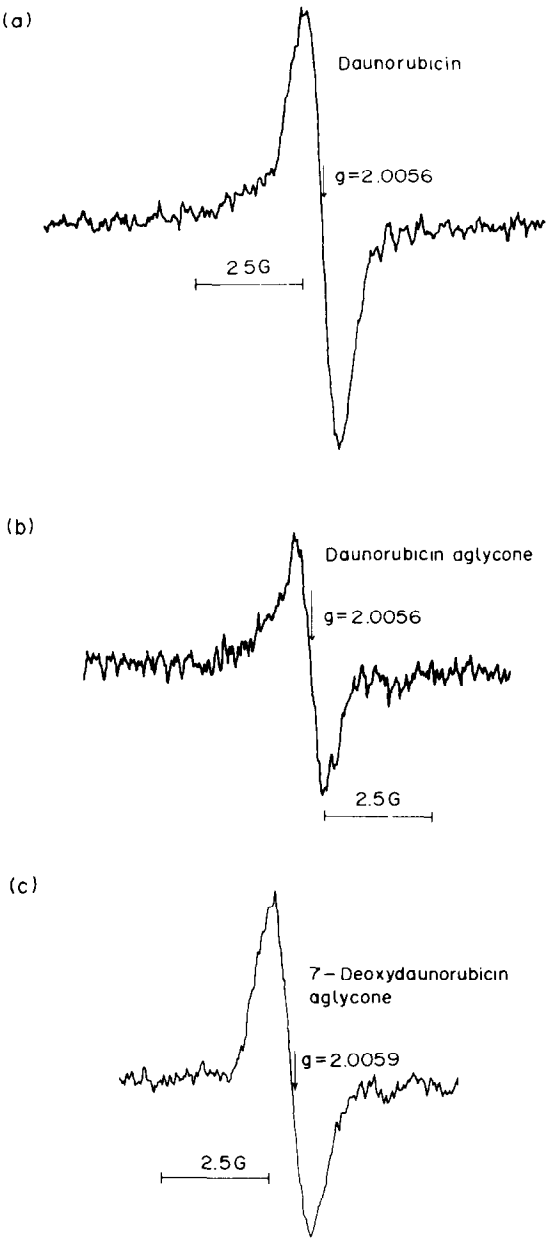


Fig. 6. EPR spectra of free radical from D_1, D_4 or dD_4 mixed with KO_2 crown ether complex in DMSO. (a) Reaction mixture contained 1.9×10^{-4} M D_1 and 5.3×10^{-4} M KO_2 crown ether complex in DMSO. Modulation amplitude and receiver gain for spectrometer were 1.0 G and $6.3 \times 10^3 \times 10$ respectively. (b) Reaction mixture contained 1.9×10^{-4} M D_4 and 5×10^{-4} M KO_2 crown ether complex in DMSO. Modulation amplitude and receiver gain are 1.0 G and $1 \times 10^4 \times 10$. (c) Reaction mixture contained 5×10^{-5} dD_4 and 5×10^{-4} M KO_2 crown ether complex in DMSO. Modulation amplitude and receiver gains are 0.05 G and $8 \times 10^3 \times 10$.

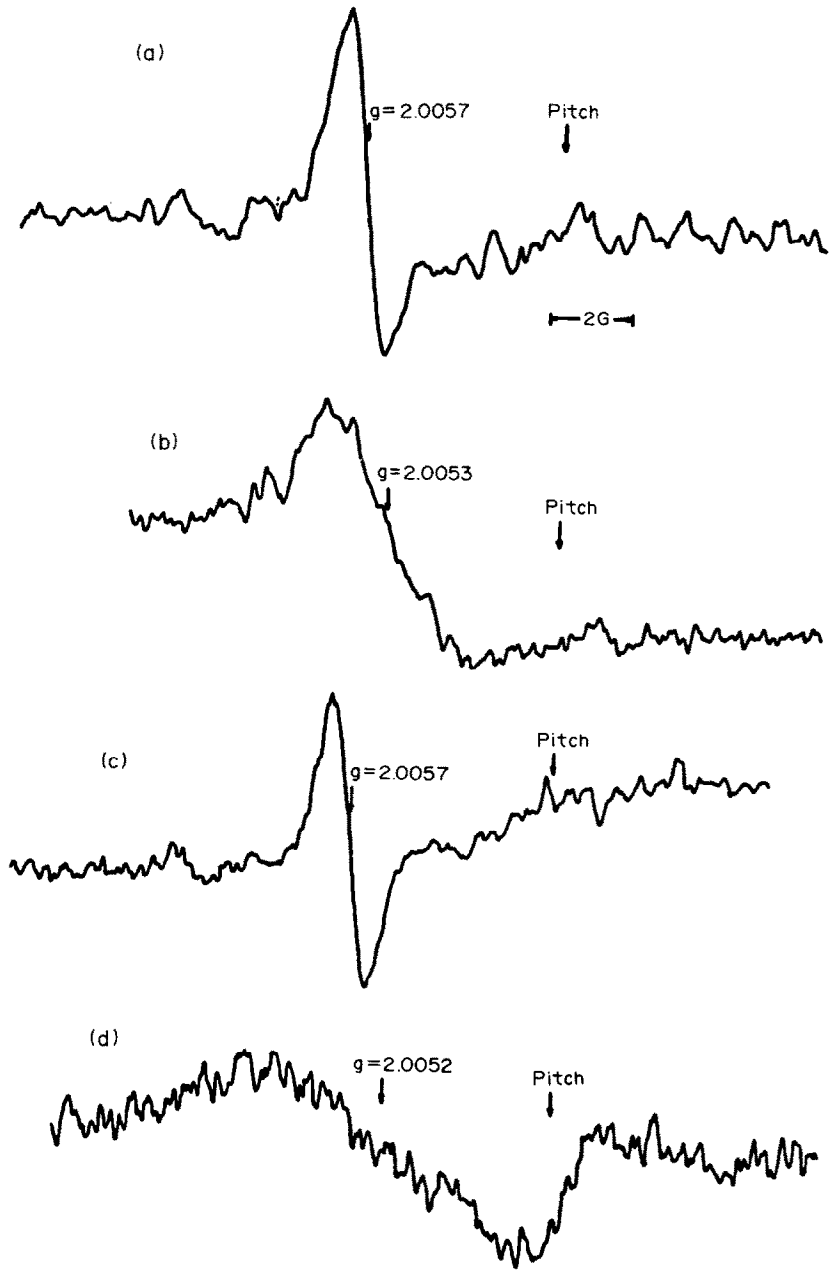


Fig. 7. EPR spectra of D_1 species in DMF (0.1 M TEAP). Scan conditions were : 5 G/min scan rate, 10 mW, incident microwave power 1 sec time constant. (a) D_1 from aerobic electrochemical reduction, 1 G modulation amplitude, $2 \times 10^4 \times 10$ gain. (b) Same as (a) plus 10 μl of 10 N NaCl, 4 G modulation, $2 \times 10^4 \times 10$ gain. (c) Same as (a) plus 10 μl H_20. (d) One milliliter of 0.12 mM D_1 and 10 μl of 1 N NaOH, 10 G modulation, $1.6 \times 10^4 \times 10$ gain.

Finally, we measured the oxygen production in these reactions. To monitor the oxygen production during superoxide anion oxidation, we modified a Clark-type oxygen electrode for use with polar aprotic solvents. Replacing saturated KCl with 2 M tetrabutylammonium perchlorate in DMSO between the electrode and Teflon membrane gave stable measurements of dissolved oxygen in the aprotic solvent. The oxygen production was stimulated when 9.5×10^{-4} M (final concentration) KO_2 crown ether complex was introduced into the reaction vessel which contained 4.8×10^{-4} M D_1, D_4 or dD_4 in DMSO (Fig. 8). The oxygen production of a 10^{-3} M KO_2 crown ether complex was linearly increased by increasing concentrations of D_1 from 10^{-4} to 5×10^{-4} M (data not shown). Stoichiometric efforts of oxygen production were also obtainable when 2×10^{-4} M D_1 in DMSO was treated with increasing concentrations of the KO_2 crown ether complex (data not shown). The addition of KO_2 crown ether com-

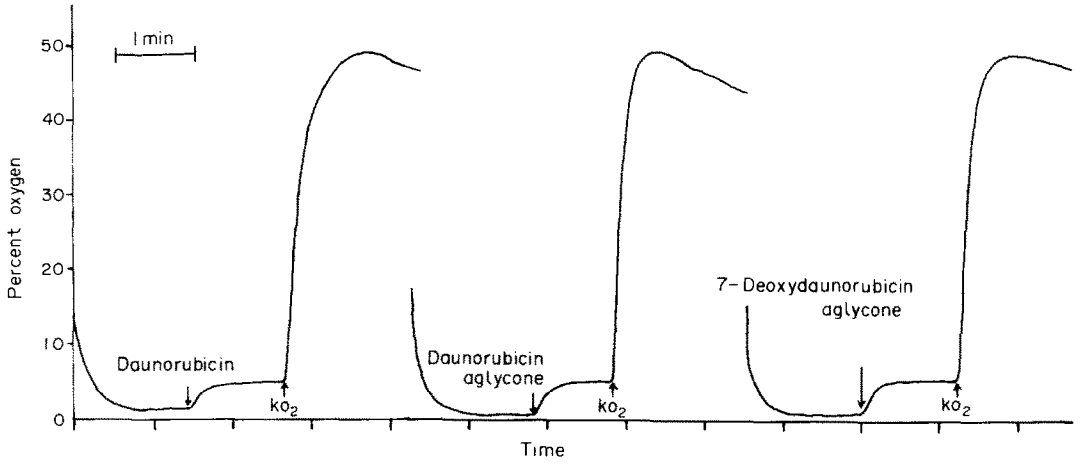


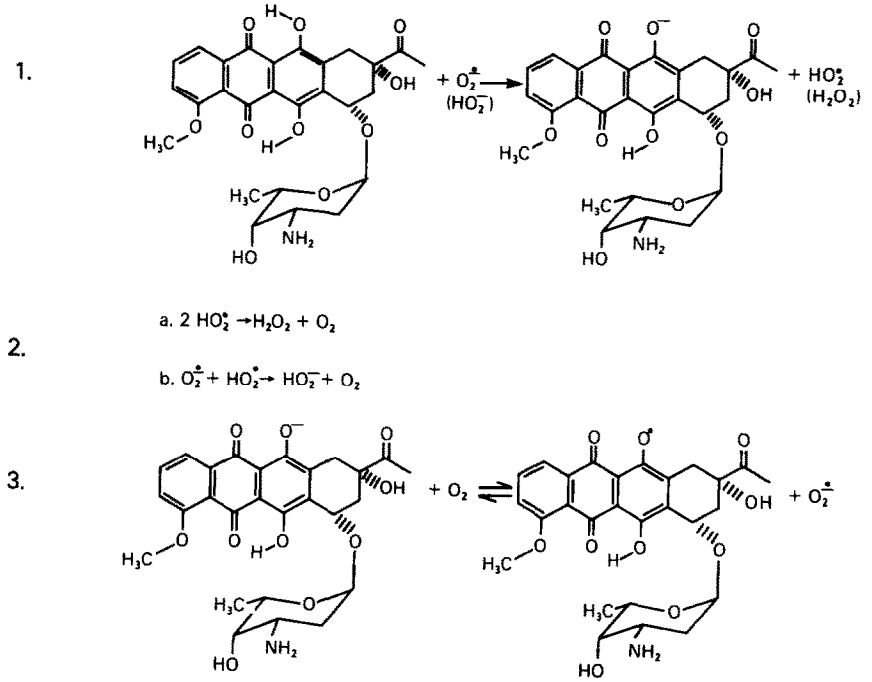
Fig. 8. Oxygen production by 4.8×10^{-4} M D_1, D_4, and dD_4 before and after 9.5×10^{-4} M KO_2 crown ether complex addition in DMSO. Conditions for the reaction are described in Materials and Methods.

plex alone to DMSO caused only a slight increase in oxygen production. This may be attributed to the natural decomposition of superoxide anion by traces of water as shown in the following equation [37]:

$$2KO_2 + H_2O = 2K^+ + {}^-OH + HOO^- + O_2$$

DISCUSSION

Numerous studies have observed that superoxide can reduce quinones in aprotic media [32, 38, 39]. However, the chemical properties of superoxide are diverse, and it can also act as an oxidant [32, 40, 41], nucleophile [32, 41–43], and a very strong base [32, 44]. The tendency of superoxide to effect proton removal from substrates and solvents is its most dominant reaction characteristic [32, 44]. Thus, besides the possible reduction of the quinone moiety of anthracyclines by superoxide, there also exists the more likely event of proton abstraction from one of the weakly acidic phenolic groups at C-6 or C-11 of the anthracyclines. Poupko and Rosenthal [38], in their study of the reduction of quinones by crown-ether solubilized potassium superoxide in DMSO, stated that hydroxy substituted benzoquinones and anthraquinones reacted spontaneously with potassium superoxide to give a color change, release of oxygen, and no EPR-detectable paramagnetic



Scheme 1. Reaction sequence for the interaction of super- oxide anion with daunorubicin in aprotic solvents.

species. These observations were attributed to the formation of the colored potassium phenolates and disproportionation of the perhydroxyl radical to release molecular oxygen:

$$2HO_2 = H_2O_2 + O_2$$

Detailes studies by Sawyer *et al.* [32, 41, 45] elucidated a mechanism for the oxidation of 3,5-di-*tert*-butylcatechol by superoxide in acetonitrile and DMF. The steps involved initial proton abstraction to form the catechol anion, disporportionation of perhydroxyl radical to give H_2O_2 and O_2, and a multistep oxidation of the catechol anion by molecular oxygen to the 3,5-di-*tert*-butyl-*o*-quinone that proceeds through a semiquinone intermediate. An identical mechanism explains the phenomena observed when the anthracyclines or quinizarin were reacted with superoxide in the aprotic solvents DMSO and DMF (Scheme 1). As Scheme 1 shows, the blue phenolate anion is formed by proton removal by superoxide (Step 1). Subsequent dismutation of perhydroxyl radical generates molecular oxygen (Step 2). Measurement of oxygen production by a modified Clark-type electrode showed that the superoxide radical was readily oxidized into oxygen by the anthracyclines. This stimulation of oxygen production by anthracyclines was stoichiometrically dependent on the concentration of both the drugs and KO_2. The molecular oxygen generated in Step 2 can then oxidize the phenolate anion to the semiquinone (Step 3). The phenolate anion had absorbance maxima at 607 and 646 nm. The semiquinone was accounted for by the EPR signal at g = 2.0057. The low EPR signal intensity of the semiquinone indicates that the equilibrium of Step 3 lies principally to the left. This is reasonable, since the oxidation potential for this step is high due to the quinone in the adjacent C ring. An even higher oxidation potential also prevents the oxidation of the semiquinone to the quinone in the B ring, as seen with catechols [41, 45]. This mechanism also accounts for the parallel phenomena observed when D_1 or quinizarin in DMF (0.1 M TEAP) was treated with NaOH, KOH, or tetrabutylammonium hydroxide. Here the EPR signal arises from oxidation of the phenolate anion by dissolved molecular oxygen from air. This mechanism also accounts for the literature report of the appearance of a low intensity free radical signal when doxorubicin is treated with base in aqueous media [36].

The immediate addition of chloroform and water to the blue-colored product returned the original reddish color. TLC showed that these products have the same R_f value as each parent anthracycline. This indicated that the reaction between KO_2 and anthracyclines was reversible. However, absorbance at 604–652 nm decreased with time and became colorless after setting for more than 1 hr. The identification of the colorless product(s) generated by degradation of the phenolate anion is unknown at present.

The absorbance spectra of the phenolate anions and EPR spectra of the semiquinones of D_1 and DHAQ were subject to considerable salt effects. When sodium ion was added, the EPR signal of the B-ring semiquinones shifted from g = 2.0057 to g = 2.0053. The interaction of diamagnetic metal ions with semiquinones and radical anions has been well documented. Dicationic metal ions affected the g values and hyperfine couplings of 3,4-dihyroxyphenylalanine radical anions [46]. A detailed theoretical treatment of the effect of alkali metal cations on the hyperfine couplings and g values of radical anions (including anthraquinone) in aprotic solvents showed excellent agreement with experimental values when the ion pair systems were considered to be tight (contact) ion pairs [47]. The presence of potassium ion affects the EPR spectra of quinones reduced with KO_2, an effect which could be eliminated by addition of a small amount of water to displace the cation from its close proximity to the radical anion [38]. Thus, it was not unusual that the EPR spectrum of D_1 B-ring semiquinones had slightly different G values depending on whether they were generated by reaction with superoxide in DMF (0.1 M TEAP), i.e. $(C_2H_5)_4N^+O_2^-$, or with NaOH in DMF (0.1 M TEAP). The conversion of the former spectrum to the latter by addition of NaCl confirmed the equivalency of these species.

The effects of salts on the absorbance spectra were much more dramatic. The presence of sodium ions caused a large blue shift (30–35 nm) of the absorbance of DHAQ and D_1 phenolate anions. Potassium ion caused a large blue shift (15 nm) that could be eliminated by complexation with a crown ether. Tetrabutylammonium hydroxide gave a spectrum that was virtually identical to the superoxide generated spectrum. This is consistent with a study that showed that the half-wave potential of anthraquinone in DMF was affected by complex formation with cations according to the size of the cation, i.e. Li^+ shifted the potential more positive than did $(C_2H_5)_4N^+$ [48]. Water alone caused a blue shift in the absorbance spectra of anthraquinone radical anion and dianion [49]. The origin of the blue shift in the absorbance spectrum was deciphered by McClelland [50] with ketyls. McClelland showed that the cation was localized near the oxygen atom as a contact ion-pair surrounded by a solvation shell, that there was an increase in the blue shift as the radius of the cation decreased, and that this shift was proportional to $1/r$ where r is the distance from ketyl to the center of the cation. The quantum-mechanical basis of the blue shift arises from a movement of charge away from the carbonyl group when the molecule is electronically excited. The electrostatic pertubation caused by the cation is thus less when the molecule is in the excited state than in the ground state; the minimum of the excited state potential energy curve moves to the left relative to the ground state minimum and, since the transition is vertical, a blue-shift is observed.

In conclusion, we have characterized the reaction of superoxide with the clinically important anticancer agent daunorubicin and two of its aglycones in aprotic solvents. The principal reaction was removal of a phenolic proton to generate the phenolate anion. This anion then decomposed to an unknown product(s). Since daunorubicin has considerable lipid solubility, the study of this interaction in aprotic solvents may have clinical relevance. Daunorubicin and other anthracyclines are known to generate

superoxide after their reduction by flavo-enzymes [1, 6–8]. The superoxide thus generated would necessarily be in close proximity to the anthraquinone nucleus and have an ideal opportunity to react directly by abstracting a phenolic proton. This would be particularly true if the initial interaction of oxygen with the anthracycline radical anion occurred in the lipid bilayer of a membrane or other hydrophophic region of the cell (such as chromatin) where the competitive reaction of superoxide with cytosolic water or superoxide dismutase would be minimized. Alternatively, the encounter of an anthracycline molecule with endogenous superoxide [11–13] in an aprotic arena *in vivo* could lead to the same result, i.e. generation of the anthracycline phenolate anion. Lynch and Fridovich [51] demonstrated that superoxide can easily move across erythrocyte membrane *in vitro*, particularly when the superoxide is generated adjacent to the inner membrane surface. A similar flux of superoxide through cellular membranes *in vivo* may thus provide a steady source of superoxide that could interact with membrane associated anthracyclines to generate the anthracycline phenolate anion. The role this species or its degradation product(s) plays in eliciting the antitumor or toxic effects of this class of antibiotic is unknown.

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