Kinetics and Mechanism of the Reactions of Superoxide Ion in Solution. Part 6.[1] Interaction of Superoxide Ion with Adriamycin in Aprotic and Protic Media

Igor B. Afanas'ev* and Natalia I. Polozova
All-Union Vitamin Research Institute, Nauchny proezd 14A, 117246 Moscow, U.S.S.R.

The interaction of superoxide ion with adriamycin has been studied in aprotic and protic solutions by the use of electronic absorption and n.m.r. spectroscopy. It was found that superoxide ion reacted with adriamycin by one-electron transfer in both aprotic and protic media (in the latter case against the reduction potential) due to irreversible transformation of the adriamycin semiquinone *via* dimerisation and deglycosation. The one-electron mechanism was confirmed by electrochemical reduction of adriamycin and by its reduction with $NaBH_4$, benzosemiquinone, and ascorbate. In both aprotic and protic media one-electron reduction of adriamycin by superoxide ion was favoured over deprotonation. The possible application of this mechanism to the reaction of $O_2^{-\cdot}$ with other anthraquinones was also considered.

Free-radical reactions of adriamycin ($AdrH_2$) and other anthracycline ($AnthH_2$) antibiotics have been much studied[2] in connection with the suggestion that both the anti-cancer effects[3,4] and the cardiotoxicity[5] of these antibiotics may be accounted for by the generation of oxygen and semiquinone free radicals. It has been shown[4-8] that the incubation of anthracycline antibiotics with microsomes and mitochondria leads to a sharp increase in superoxide production, which was explained by the one-electron oxidation of antibiotic semiquinones by molecular oxygen [equation (1)].

$$AnthH_2^{-\cdot} + O_2 \rightleftharpoons AnthH_2 + O_2^{-\cdot} \qquad (1)$$

Chemical studies are not in full agreement with this proposal. Thus, although pulse-radiolysis studies[9,10] show that in aqueous solution equilibrium (1) must indeed be shifted to the right (K_1 *ca.* 10^3),[10] we[11] have previously found that in aprotic media (DMF) superoxide ion reacts with adriamycin practically irreversibly. We assumed that the reaction proceeds *via* a one-electron transfer mechanism to form the adriamycin semiquinone which at the next stage forms a dimeric or oligomeric diamagnetic complex. Recently, our conclusion was questioned.[12,13] The authors of these papers argued that $O_2^{-\cdot}$ reacts with anthracycline antibiotics *via* the deprotonation reaction (2) and not *via* the one-electron transfer (−1).

$$O_2^{-\cdot} + AnthH_2 \longrightarrow HO_2^{\cdot} + AnthH^- \qquad (2)$$

We re-investigated the interaction of superoxide ion with adriamycin in aprotic media (acetonitrile) and extended the study to water–acetonitrile solutions with a water content up to 90%. Acetonitrile was chosen as the aprotic solvent instead of dimethylformamide because in this solvent $O_2^{-\cdot}$ has a strongly marked maximum at 249 nm.[14]

Results

Reaction of Superoxide Ion with Adriamycin.—Under all conditions studied, *i.e.* in water–acetonitrile solutions with a water content of 0—90% v/v, superoxide ion reacted quantitatively with adriamycin. In all cases the spectra of product X in the visible region had a similar form (Figure 1), but the positions of the maxima depended on the water content: two maxima of the product were at 552 ± 2 and 593 ± 2 nm when the water content was 23—90% and they shifted up to 587.5 and 623 nm in pure acetonitrile (see Table). In acetonitrile the product X was rather unstable, its spectrum disappearing after

Table. Absorption maxima of adriamycin and product X obtained in the reaction with $O_2^{-\cdot}$ in water–acetonitrile solution ([adriamycin] 0.2—0.3 × 10^{-4}M)

	$\lambda_{max.}$/nm			
H_2O	Adriamycin		Product X	
% v/v				
0.0	474	496	587.5	623
2.0	475	496	574	608
4.8	475.5	497	566.5	598.5
23.3	478	495	555	593
58.6	477	494	553	590
85.0	479	496	551	591
100.0[a]	479	498	549.5	589.5

[a] Obtained for the reaction of adriamycin with NBu_4OH.

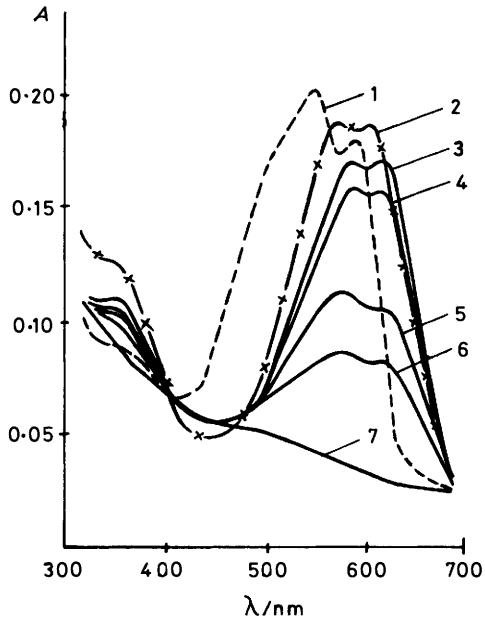


Figure 1. Absorption spectra of the product of reaction of adriamycin with $O_2^{-\cdot}$ (product X) in water–acetonitrile solutions (10 mm cell): 1, 90% v/v water, [$AdrH_2$] 0.19 × 10^{-4}M, [$O_2^{-\cdot}$] 2.85 × 10^{-4}M; 2, 2% v/v water, [$AdrH_2$] 0.17 × 10^{-4}M, [$O_2^{-\cdot}$] 4.96 × 10^{-4}M; 3, 0% v/v water, [$AdrH_2$] 0.15 × 10^{-4}M, [$O_2^{-\cdot}$] 1.61 × 10^{-4}M; 4—7, decrease in absorption after 1 h

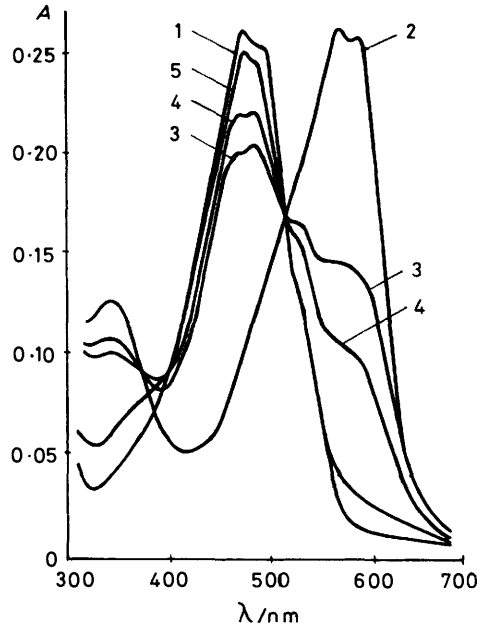


Figure 2. Electrochemical reduction of adriamycin in acetonitrile solution containing 4.8% water (10 mm cell): 1, absorption spectrum of adriamycin·HCl, [$AdrH_2$] 2.7 × 10^{-5}M; 2, absorption spectrum after completing electrolysis; 3 and 4, same spectrum after 15 and 30 min; 5, same spectrum after acidification

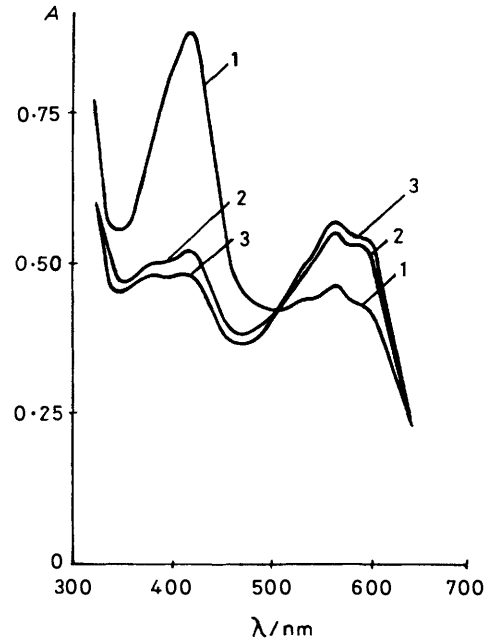


Figure 3. Interaction of adriamycin with benzosemiquinone in acetonitrile solution containing 6.5% v/v water ([$AdrH_2$] 0.62 × 10^{-4}M, [benzosemiquinone] 5.20 × 10^{-4}M, 10 mm cell); 1—3 absorption spectra 1, 2.5, and 5.5 min after mixing the reactants

1—1.5 h without the appearance of any new maxima in the visible region (Figure 1). In solutions with high water content the decay rate of the product X was considerably smaller. The spectrum of product changed insignificantly after argon or oxygen flushing. However, the spectrum immediately disappeared and a new one very similar to that of adriamycin appeared when the solution was acidified.

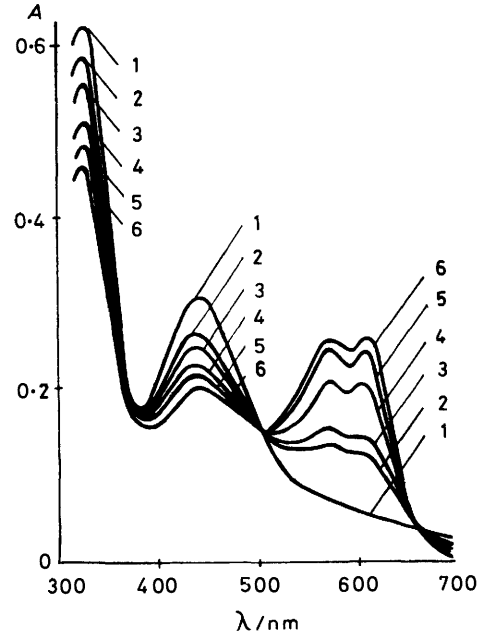


Figure 4. Reduction of adriamycin with $NaBH_4$ in dimethylformamide, [$AdrH_2$] 0.50 × 10^{-4}M, [$NaBH_4$] 0.045M, 10 mm cell; 1—6, absorption spectra 2, 3.5, 6, 12, 20, and 30 min after adding $NaBH_4$

Chemical Reduction of Adriamycin.—In order to study the mechanism of the formation of product X, we investigated the electrochemical and chemical (with $NaBH_4$) reduction of adriamycin as well as the reactions of adriamycin with tetrabutylammonium hydroxide and benzosemiquinone. Electrochemical reduction in a solution containing 4.8% water resulted in the formation of the product X (Figure 2) (time of electrolysis 45—60 s). In due course the spectrum changed, the decrease in maxima at 560—600 nm following the appearance of maxima in the region 475—540 nm.

In a similar manner adriamycin reacted with benzosemiquinone and tetrabutylammonium hydroxide. For example, mixing solutions of adriamycin and benzosemiquinone (the last being prepared from $O_2^{-\cdot}$ and an excess of benzoquinone[11]) gave the same product X (Figure 3). The reduction of adriamycin with $NaBH_4$ also led to this product but *via* the formation of an intermediate which had a maximum at 437 nm (Figure 4). It should be noted that in all cases (excluding the reaction with $NaBH_4$) acidification led to the immediate appearance of a spectrum very similar to that of adriamycin (see for example Figure 2).

Discussion

Two possible pathways for the interaction of superoxide ion with adriamycin should be considered. Both one-electron transfer reaction (3) and deprotonation reaction (4) are

$$O_2^- + AdrH_2 \rightleftharpoons O_2 + AdrH_2^{-\cdot} \longrightarrow \text{product X} \quad (3)$$

$$O_2^- + AdrH_2 \rightleftharpoons HO_2^{-\cdot} + AdrH^- \quad (4)$$

reversible processes. The one-electron reduction potential of adriamycin in aqueous solution is *ca.* 0.15 V smaller than that of dioxygen [$E_o(AdrH_2/AdrH_2^{-\cdot})$ −0.292 V[9] or −0.328 V,[10] $E_o(O_2/O_2^{-\cdot})$ = (−0.10)—(−0.15) V[12]]; therefore equilibrium (3) is shifted to the left in aqueous solution. In aprotic media the reduction potentials of hydroxyanthraquinones such as dauno-

mycinone [$E_{\frac{1}{2}}$ −0.625 V (s.c.e.)[12]] and adriamycin [$E_{\frac{1}{2}}$ −0.665 V (s.c.e.)[16]] increased significantly due to internal hydrogen bonding in semiquiones and become more positive than that of dioxygen [(−0.7)—(−0.8) V[15]]. Therefore equilibrium (3) is shifted to the right. As $HO_2^{\bullet}$ is a stronger acid than adriamycin [$pK_a(HO_2^{\bullet})$ 4.75,[17] $pK_a(AdrH_2)$ 10.1[18]], the deprotonation equilibrium (4) must be shifted to the left.

But really these thermodynamic estimates cannot determine the direction of reaction and the nature of the final product as both equilibria (3) and (4) are followed by irreversible steps. In the case of the one-electron transfer reaction (4), it is dimerisation and deglycosation of adriamycin semiquinone (see below). It is also well known that the protonation of superoxide ion is always followed by reaction (5). The formation of product

$$HO_2^{\bullet} + O_2^{-\bullet} \longrightarrow HO_2^{-} + O_2 \qquad (5)$$

X in such typical reduction processes as the electroreduction of adriamycin, reduction with $NaBH_4$, and one-electron reduction by benzosemiquinone proves that $O_2^{-\bullet}$ reacts with adriamycin in aprotic media *via* reaction (3). The thermodynamic data for aprotic media[12,16] support this conclusion. In this case it is also obvious why reaction (4) does not occur. It is well known that the rate constants for exothermic one-electron transfer reactions are *ca.* 10^8–10^9 l mol^{-1} s^{-1}. As the rate constant for reaction (4) must be *ca.* 10^4 l mol^{-1} s^{-1} (the rate constant for the deprotonation of phenol by $O_2^{-\bullet}$ in acetonitrile and DMF is 10^4 l mol^{-1} s^{-1}[19]), only reaction (3) will take place.

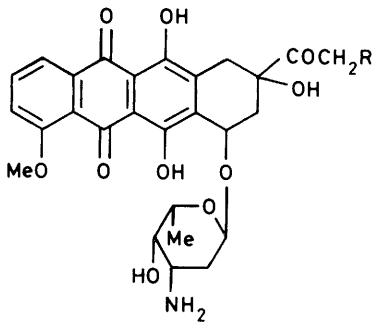
However we found that superoxide ion reacts irreversibly with adriamycin to form product X not only in neat acetonitrile but also in water–acetonitrile solutions up to 90% water (Figure 1). The one-electron transfer mechanism (3) in aqueous solution is confirmed by the fact that the same product is formed in the reaction of ascorbate anion with adriamycin in the presence of Fe^{3+}: this process can proceed only through one-electron transfer (data will be reported in detail elsewhere). It should also be noted that Kleyer and Koch[20] have shown that a similar product is formed in the reduction of daunomycin (**Ib**) [a close analogue of adriamycin (**Ia**)] by the neutral free radical 3,3,5-trimethyl-2-oxomorpholin-3-yl in protic medium (methanol).

Thus transformation of adriamycin semiquinone into the diamagnetic product X shifts equilibrium (3) to the right against a difference in reduction potential in aqueous solution, and deprotonation (4) remains incapable of competing with the one-electron transfer (3).

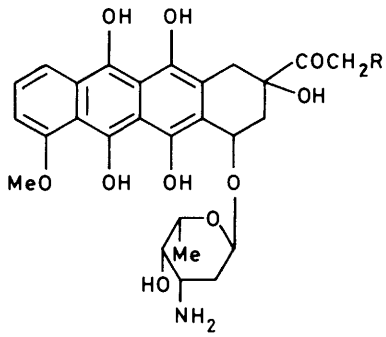
In spite of several attempts to identify product X, its structure remains uncertain. From the similarity of the absorption spectra obtained for the reactions of anthracycline antibiotics with superoxide ion and alkali, it has been concluded[12,13] that product X should be the anthracycline anion. But we have already shown that it is not true as the adriamycin anion cannot be formed in electrochemical and chemical reduction with $NaBH_4$ as well as in reactions with semiquinones and ascorbate anion. We also attempted to identify product X (see Experimental section). Unlike adriamycin, the crystalline precipitate obtained after acidification of product X was weakly soluble in water and apparently consisted of compound (**IV**) and its analogue without the $COCH_2OH$ group (a major component). Thus it is certain that during reaction with $O_2^{-\bullet}$ adriamycin loses daunosamine, and therefore there is no doubt that the reaction between $O_2^{-\bullet}$ and anthracyclines is not deprotonation.

Kleyer and Koch[20] have recently proposed a mechanism for the reduction of daunomycin (**Ib**) by 3,5,5-trimethyl-2-oxomorpholin-3-yl in methanol. They believe that at the beginning (**Ib**) is reduced to hydroquinone (**II**) which then decomposed to form a tautomer of deoxyaglycone (**III**). The latter is converted into the deoxyaglycone (**IV**) having a practically identical absorption spectrum with the antibiotic. This seems to be the reason for the 'regeneration' of the antibiotic spectrum observed by us and in ref. 20 upon acidification of the reaction solution or in the presence of proton donors.

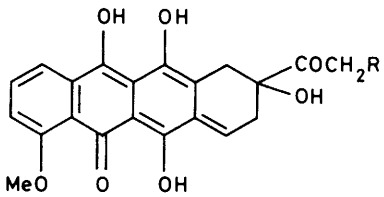
This mechanism (with some modification) can be accepted for the reaction of superoxide ion with anthracycline antibiotics



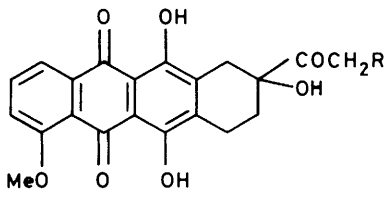
(**Ia**) R = OH
(**Ib**) R = H



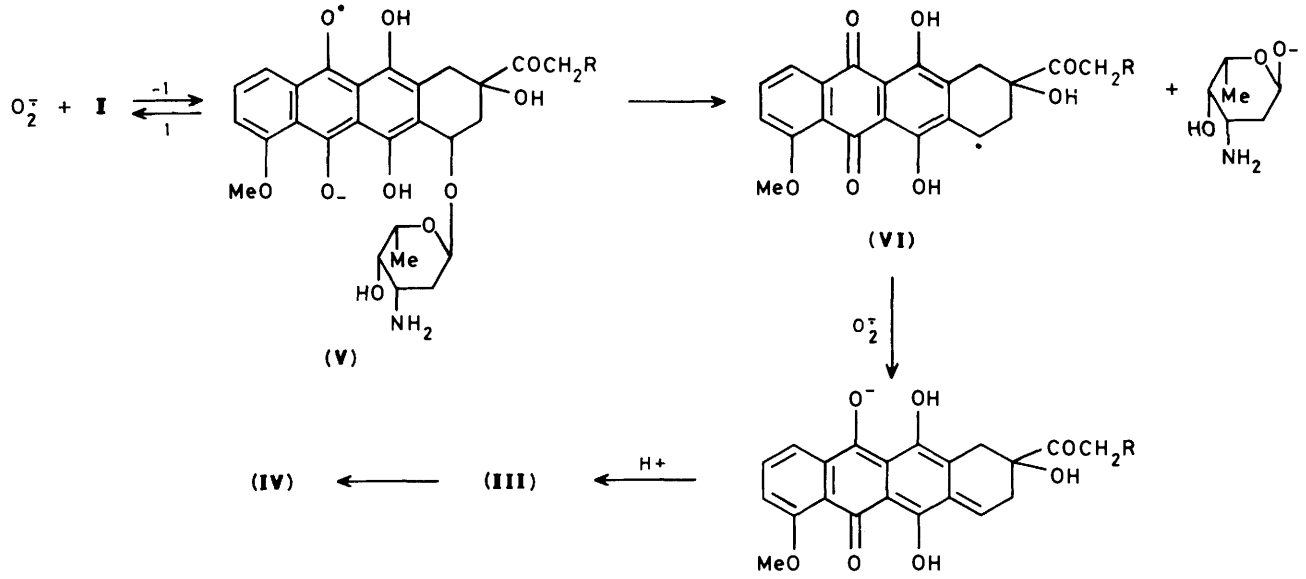
(**II**)



(**III**)



(**IV**)



Scheme.

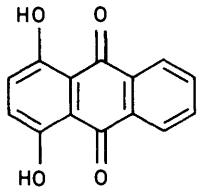
(see Scheme). It is supposed that daunosamine is removed from semiquinone (**V**) and not hydroquinone (**II**) (as superoxide ion cannot reduce adriamycin to hydroquinone, at least in aprotic media). The neutral semiquinone of deoxyaglycone (**VI**) is reduced by the second superoxide molecule into an anion of 'tautomer' (product X) which after protonation is transformed into deoxyaglycone (**IV**). It should be noted that the reductive cleavage of adriamycin with the intermediate formation of a semiquinone has been suggested earlier.[7]

In accord with this mechanism product X is not tautomer (**III**) but its anion. This is confirmed by the fact that at acidification product X is instantly converted into deoxyaglycone (**IV**). Furthermore, the absorption maxima of X are significantly shifted to the longwave region by comparison with those of adriamycin and this is more characteristic for an anion.

The spectral data obtained in mixed water–acetonitrile solvents deserve separation consideration. As may be seen from the Table, the absorption maxima of product X are shifted to the shortwave region by 32—36 nm when the water content increases from 0 to 85%. This is understandable if product X is an anion, as the absorption maxima of ions in aqueous solution are usually shifted to the shortwave region relative to those in aprotic media due to selective solvation of the basic state[21] (see, for example, refs. 22 and 23). Nakazawa and his co-workers[13] also observed the same shift for the absorption maxima of the product obtained in the reaction of KO_2 with daunomycin in DMF (604 and 652 nm) by comparison with the maxima in DMF–water (572 and 611 nm). They believe that this shift is due to the complexation of the product with Na^+ ions. But our data refute this proposal as all experiments presented in the Table were carried out in the presence only of Bu_4NClO_4.

An analogous mechanism can be proposed for the electrochemical reduction of adriamycin and for the reaction of adriamycin with semiquinones. But for the reduction with $NaBH_4$ hydroquinone (**II**) (with a maximum at 437 nm) is probably formed as an intermediate, *i.e.* the reaction proceeds in accord with the Kleyer–Koch mechanism[20] (Figure 4).*

If cleavage of the glycoside bond is the probable pathway for the transformation of the semiquinone formed in the reaction of superoxide ion with anthracycline antibiotics, such a mechanism cannot explain the fact that a product very similar to X is formed in the reaction of $O_2^{-\cdot}$ with quinizarin (**VII**) in DMF;[13] for this compound the formation of a tautomer similar to (**III**) is impossible. For quinizarin $E_{\frac{1}{2}}$ −0.558 in DMF,[16] so superoxide ion must react with (**VII**) *via* one-electron transfer.



(**VII**)

Therefore, there must be another pathway than that in the Scheme by which a semiquinone could be converted into a diamagnetic product. It should also be noted that the electrogenerated semiquinone of daunomycinone was converted into a similar diamagnetic product in the presence of dioxygen.[12]

What can the structure of product X be in this case? Earlier we proposed[11] that product X may be the semiquinone dimer. The ability of semiquinones to form dimers is well known; for example benzosemiquinone forms a dimer in ethanol at −130 °C.[24] It is difficult to explain all the experimental facts concerning the interaction of superoxide ion with various anthraquinone and anthracycline antibiotics, but as a workable hypothesis we propose that the formation of semiquinone dimer always precedes deglycosation. Then, after acidification, deoxyaglycone is formed in the case of anthracyclines (together with some other degradation products), whereas an initial quinone is formed in the case of simple anthraquinones (due to the disproportionation of neutral semiquinones with subsequent oxidation of the hydroquinone formed).

There is difficulty in interpeting the results obtained in aqueous solution. It seems that increasing the water content from 0 to 85% does not change the structure of product X. The same absorption spectrum was also obtained in the reaction of adriamycin with ascorbate and tetrabutylammonium hydroxide

* A referee pointed out that during reduction with $NaBH_4$ the $COCH_2OH$ group may also be reduced. This fact however will not affect the absorption spectrum of product X.

in water. But it is believed[25,26] that at basic pH the absorption spectra observed correspond to the anthracycline anion and dianion. However there is other evidence that the interaction of adriamycin with alkali is not a simple deprotonation. Thus Chinami and his co-workers[27] have shown that in this reaction adriamycin semiquinone is formed. They believe that this semiquinone is formed as a result of the oxidation of the adriamycin anion by dioxygen [reaction (6)].

$$AdrH^- + O_2 \longrightarrow AdrH^{\bullet} + O_2^{-\bullet} \qquad (6)$$

The superoxide ion formed will react with another adriamycin molecule giving product X, whereas $AdrH^{\bullet}$ will disproportionate to form the corresponding hydroquinone and quinone. As shown, adriamycin hydroquinone is rapidly oxidised to form product X. It is of interest that there may be other pathways to product X in basic solution. For example, we found that even benzoquinone (without a hydroxy group and therefore not able to form an anion) immediately forms benzosemiquinone in alkaline solution. Furthermore, it has recently been shown[28] that anthraquinone reacts with alkali (Bu_4NOH or NaOH) in acetonitrile apparently *via* addition of HO^- to the carbonyl group with the subsequent interaction of an adduct with a second quinone molecule to form the semiquinone.

Thus we conclude that superoxide ion reacts with adriamycin and apparently with other anthracycline antibiotics *via* a one-electron transfer mechanisn in both aprotic and protic media. At the beginning the semiquinone formed then gives a diamagnetic dimer which decomposes to yield, as final product, deoxyaglycone. Such processes may be of importance for biological systems as they may be relevant to the anti-cancer effect and cardiotoxicity of the anthracycline antibiotics.

Experimental

Absorption spectra were recorded on a Cary 219 spectrophotometer. N.m.r. spectra were measured on a Bruker WP200 spectrometer.

Adriamycin hydrochloride (Farmatalia) was used. Solutions of superoxide ion in acetonitrile were prepared by electrochemical reduction of molecular oxygen in the presence of tetrabutylammonium perchlorate.[1] The half-life of superoxide ion was 30—35 h.[14] Reactions of adriamycin with $O_2^{-\bullet}$, $NaBH_4$, NBu_4OH, and benzosemiquinone were carried out in the 2 and 10 mm quartz cells.

Analysis of Products obtained after Acidification of Product X.—Aqueous solution of product X prepared by alkali treatment of adriamycin was acidified and the red crystals formed were separated by centrifugation at pH 7. The precipitate was rinsed with water several times to remove the water-soluble products and dried over P_2O_5, m.p. 180—190 °C, R_F 0.45 (Silufol plates, 20:3:5:4 chloroform–methanol–acetic acid–water) (principal spot) (adriamycin in the same system has R_F 0.1); δ (200 MHz, [$^2H_6$]Me_2SO) 2.18 (2 H, m, 8-H), 2.74—3.02 (3 H, m, 7- and 10-H), 3.99 (3 H, s, OMe), 5.55 (1 H, s, 9-OH), 7.64 (1 H, m, 3-H), 7.90 (2 H, m, 1- and 2-H), 13.25 (1 H, s, phenolic OH), and 13.81 (1 H, s, phenolic OH). The n.m.r. spectrum did not contain signals corresponding to daunosamine [for adriamycin, δ 1.17 (3 H, s, 6′-H), 1.80 (2 H, m, 2′-H), 2.17 (2 H, m, 8-H), 2.75 (1 H, d, 10β-H), 2.99 (1 H, d, 10α-H), 3.50 (2 H, m, 3′- and 4′-H), 3.99 (3 H, s, OMe), 4.17 (1 H, m, 5′-H), 4.57 (2 H, s, 14-H), 4.98 (1 H, s, 7-H), 5.30 (1 H, s, 1′-H), 5.45 (1 H, s, 9-OH), 7.65 (1 H, m, 3-H), 7.90 (2 H, m, 1- and 2-H), 13.22 (1 H, s, phenolic OH), and 14.00 (1 H, s, phenolic OH)]. These n.m.r. spectra may be compared with those of adriamycin and 7-deoxyaglycone reported by Smith *et al.*[29]

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Received 30*th April* 1986; *Paper* 6/841