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MECHANISM OF THE INTERACTION OF SUPEROXIDE ION AND ASCORBATE WITH ANTHRACYCLINE ANTIBIOTICS

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The interaction of superoxide ion and ascorbate anion with anthracycline antibiotics (adriamycin and aclacinimycin A) as well as with their Fe^{3+} complexes has been studied in aprotic and protic media. It was found that both superoxide and ascorbate reduce anthracyclines to deoxyaglycons *via* a one-electron transfer mechanism under all conditions studied. The reaction of ascorbate anion with adriamycin and aclacinomycin A in aqueous solution proceeded only in the presence of Fe^{3+} ions; it is supposed that an active catalytic species was Fe^{3+} adriamycin. It is also supposed that the reduction of anthracycline antibiotics by $O_2^{\cdot-}$ and ascorbate in cells may increase their anticancer effect.

KEY WORDS: Superoxide, ascorbate, adriamycin, aclacinimycin A.

INTRODUCTION

Recently, much attention has been given to studying free radical reactions of anthracycline antibiotics and their metal complexes. It was shown[1–4] that the incubation of anthracycline antibiotics with mitochondria and microsomes results in the generation of superoxide ion supposedly *via* the direct one-electron transfer to dioxygen.

$$\text{Anth}^{\cdot-} + O_2 \rightleftharpoons \text{Anth} + O_2^{\cdot-} \quad (1)$$

This proposal was confirmed by pulse-radiolysis studies[5,6] which showed that equilibrium 1 is indeed shifted to the right in water (K_1 is about 10^3).[6] Contrary to that $O_2^{\cdot-}$ reacted irreversibly with adriamycin in DMF and acetonitrile[7,8] as it should be expected from comparison of the reduction potentials of anthracyclines and dioxygen in aprotic media.[9] On the other hand it has been shown[10–13] that adriamycin can generate oxygen radicals *via* another pathway, namely as a result of the reduction of O_2 by their iron complexes.

$$Fe^{2+}\text{-Anth} + O_2 \rightleftharpoons Fe^{3+}\text{-Anth} + O_2^{\cdot-} \quad (2)$$

$$\text{or } Fe^{3+}\text{-Anth} \rightleftharpoons Fe^{2+}\text{-Anth}^{\cdot+} \quad (3)$$

$$Fe^{2+}\text{-Anth}^{\cdot+} + O_2 \rightleftharpoons Fe^{3+}\text{-Anth}^{\cdot+} + O_2^{\cdot-} \quad (4)$$

$$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (5)$$

$$O_2^{\cdot-} + H_2O_2 \xrightarrow{Fe^{2+}} HO\cdot + HO^- + O_2 \quad (6)$$

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Reactions 1, 2, and 4 are usually considered as the modes of anticancer and cardiotoxic effects of anthracyclines. But it was also assumed[14] that anthracyclines can manifest their anticancer effect *via* the formation of active intermediates during reduction. Earlier, we have proposed[7,8] that such active intermediates as semiquinone dimers and deoxyaglycon tautomers are formed during the interaction of superoxide ion with adriamycin. Now, we studied the interaction of two important physiological reductants, O_2^- and ascorbate, with two anthracycline antibiotics, adriamycin (Adr) and aclacinomycin A (Acl) as well as with their iron complexes in both aprotic media (acetonitrile or dimethylformamide) and aqueous solution.

MATERIALS AND METHODS

Adriamycin hydrochloride and aclacinomycin A hydrochloride of commercial purity were used. Solutions of superoxide ion in acetonitrile or DMF were prepared by electrochemical reduction of molecular oxygen in the presence of tetrabutylammonium perchlorate (the supporting electrolyte).[7] The cell had a mercury cathode and a platinum anode separated by a cock. Acetonitrile was dried by refluxing over P_2O_5 during 15–20 h and was twice distilled over anhydrous K_2CO_3. DMF was dried over anhydrous K_2CO_3 and was twice vacuum distilled. Solutions of superoxide ion (0.001–0.01 M) were freshly prepared prior to each experiment. (A half-time of O_2^- was equal to 30–35 h). Solutions of ascorbate anion were prepared by neutralization of the solutions of ascorbic acid (commercial purity) in acetonitrile and water with tetrabutylammonium hydroxide. Fe^{3+}-adriamycin was prepared from $(0.5–1)\cdot10^{-3}$ M solutions of antibiotic and $(2–5)\cdot10^{-3}$ M solutions of ferric chloride in acetonitrile and water (pH 2.6–2.8).

Reactions of superoxide ion or ascorbate with anthracycline antibiotics or their iron complexes were carried out in the 2 mm quartz cells. Absorption spectra were recorded on a Cary 219 spectrophotometer.

RESULTS

Superoxide ion reacted quantitatively with adriamycin and aclacinomycin A under all conditions studied i.e. in pure acetonitrile and mixed acetonitrile-water solutions up to 90% water content. In all cases the absorption spectra of products were shifted to the long wave region relatively parent antibiotics (see Table I) and depended strongly on the solvent. (Spectra of the reaction product obtained in reaction of O_2^- with Acl are presented in Fig. 1; analogous spectra for the reaction with Adr were given.[6] The treatment of reaction mixture with acid led to the disappearance of product spectrum and the appearance of a new one very similar to that of parent antibiotic.

The same products were obtained during the treatment of Adr in DMF and Acl in acetonitrile with ascorbate anion, but in this case the reactions did not proceed to completion (Fig. 2). In aqueous solution the reaction of ascorbate anion with anthracyclines appears to proceed only in the presence of Fe^{3+} ions as EDTA inhibited the process (Fig. 3). Fe^{3+}-Adr reacted with ascorbate anion in aqueous solution and with O_2^- in acetonitrile to form apparently the same product as that obtained in the reactions with Adr (Figs. 4 and 5). But in acetonitrile the reaction of Fe^{3+}-Adr with ascorbate anion led to the formation of a new product with a maximum at 486 nm.

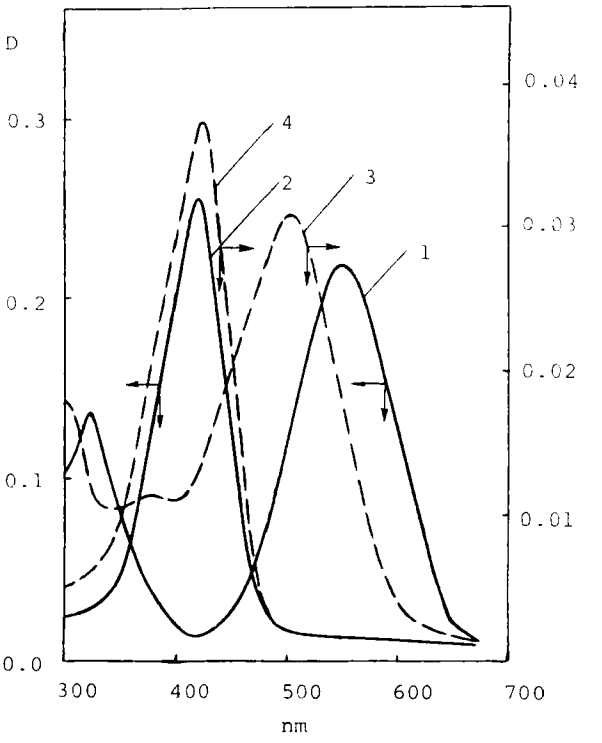


FIGURE 1 The interaction of O_2^- with aclacinomycin A. 1. Spectrum of the product in acetonitrile, [Acl] = $1.19 \cdot 10^{-4}$ M, [O_2^-] = $2.7 \cdot 10^{-4}$ M. 2. The same after acidification. 3. Spectrum of the product in water-acetonitrile solution (90% of water), [Acl] = $1.9 \cdot 10^{-5}$ M, [O_2^-] = $3.5 \cdot 10^{-4}$ M. 4. The same after acidification.

TABLE I

Absorption maxima of anthracycline antibiotics and reaction products

	λ_{max} (nm)			
	acetonitrile		water	
Adr	474	496	479	498
Acl	430		430	
Fe^{3+}-Adr	500	600 (2% of H_2O)	496	600
Fe^{3+}-Aci	476[a]		477[a]	
O_2^- + Adr	587.5	623	551	591 (15% of MeCN)
	555	593 (23.3% of H_2O)		
AH^- + Adr	552	598 (in DMF)		598
AH^- + Acl	558		513	
O_2^- + Fe^{3+}-Adr	552	597 (25% of H_2O)		
AH^- + Fe^{3+}-Adr	486	(0.4% of H_2O)	545	600

[a] Differential spectrum

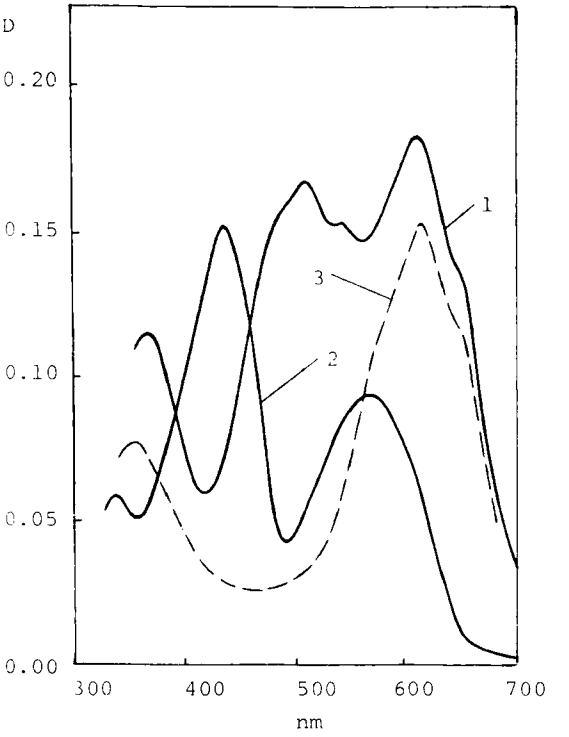


FIGURE 2 The interaction of ascorbate anion with adriamycin and aclacinomycin A. 1. (AH^- + Adr) in DMF, [Adr] = $1.15 \cdot 10^{-4}$ M, [AH^-] = $5.5 \cdot 10^{-4}$ M. 2. (AH^- + Acl) in acetonitrile, [Acl] = $1.44 \cdot 10^{-4}$ M, [AH^-] = $4.91 \cdot 10^{-4}$ M. 3. Spectrum of the product obtained in the reaction of O_2^- with Adr in DMF.

DISCUSSION

We have previously proposed[7,8] that superoxide ion reacts with adriamycin *via* an one-electron transfer mechanism to form a semiquinone dimer or the tautomer anion of deoxyaglycon. Our proposal was questioned in recent works[15,16] in which was assumed that the product formed is the adriamycin anion. But we have already shown[8] that the same product was formed in electrochemical reduction of adriamycin and in the reactions of adriamycin with benzosemiquinone and $NaBH_4$: all these reactions are typical reduction processes in which the deprotonation of adriamycin seems to be impossible. Furthermore, recent determination of the reduction potential of adriamycin in DMF ($E_{1/2} = -0.665$ V(s.c.e.))[9] confirmed that the one-electron reduction of adriamycin by superoxide ion ($E_{1/2}(O_2/O_2^-) = (-0.7) - (-0.8)$ V(s.c.e.)[17]) is an exothermic process, and so its rate constant should be about $10^9\,M^{-1}\,s^{-1}$ (as this reaction must proceed *via* an outer-sphere one-electron transfer mechanism).

Now we studied the interaction of two anthracycline antibiotics, adriamycin and aclacinomycin A, with superoxide ion and ascorbate anion in aprotic and protic

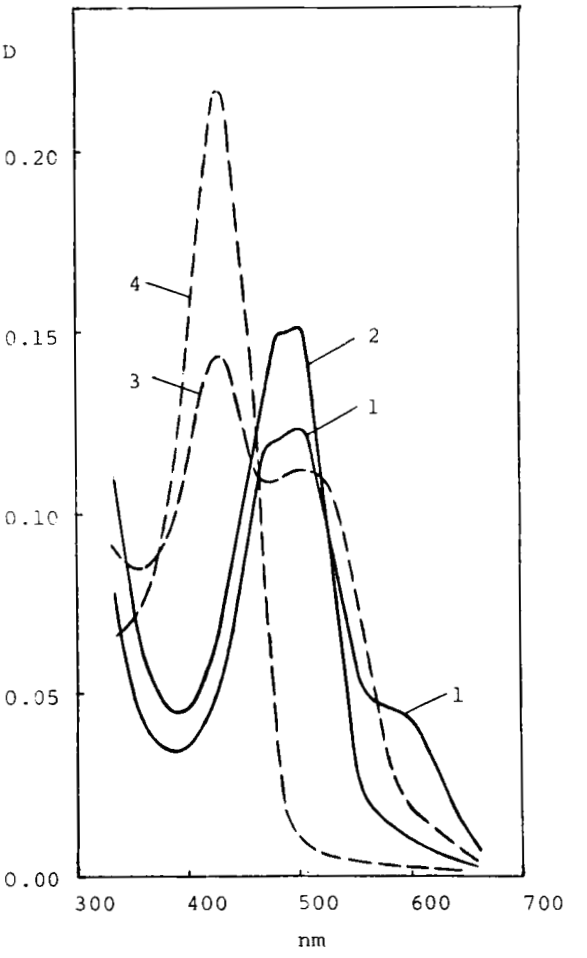


FIGURE 3 The interaction of ascorbate anion with adriamycin and aclacinomycin A in water in the presence of Fe^{3+} ions. 1. (AH^- + Adr) [Adr] = $6.3 \cdot 10^{-5}$ M, [AH^-] = $2.47 \cdot 10^{-3}$ M, [$FeCl_3$] = $2.7 \cdot 10^{-4}$ M. 2. The same in the presence of EDTA ($4.0 \cdot 10^{-4}$ M). 3. (AH^- + Acl) [Acl] = $7.7 \cdot 10^{-5}$ M, [AH^-] = $3.07 \cdot 10^{-3}$ M, [$FeCl_3$] = $7.9 \cdot 10^{-5}$ M. 4. The same in the presence of EDTA ($3.3 \cdot 10^{-4}$ M).

media. Our results obtained in the experiments with ascorbate anion strongly confirm the one-electron transfer mechanism of the interaction of superoxide ion with anthracycline antibiotics. The reaction of ascorbate anion with adriamycin in DMF results in the same product that was obtained in the reaction with $O_2^{\cdot-}$.[7] It is evident that ascorbate anion AH^- (pK_aAH_2) = 4.25[18]) cannot deprotonate adriamycin (pK_a(Adr) = 9.01[19]) therefore the one-electron transfer reactions occur in both cases.

The reactions of anthracyclines with ascorbate anion are apparently more slow processes than those with superoxide ion (compare Figs. 1 and 2). But it should be noted that the reactions with ascorbate anion were always carried out with a small

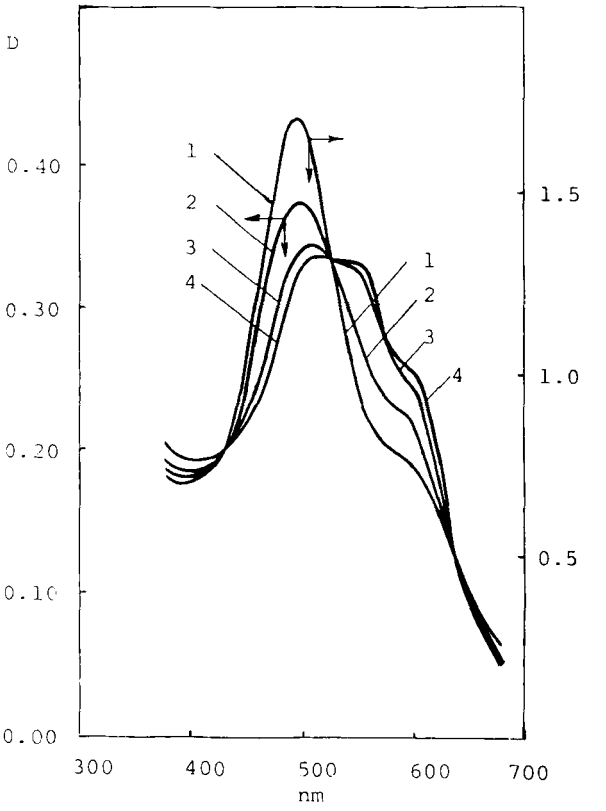
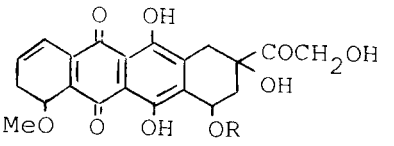


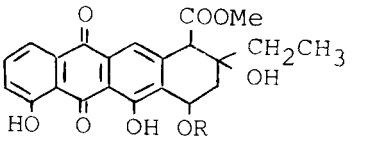
FIGURE 4 The interaction of ascorbate anion with Fe^{3+}-Adr in water. 1. Spectrum of Fe^{3+}-Adr, [Adr] = $0.98 \cdot 10^{-3}$ M, [$FeCl_3$] = $2.2 \cdot 10^{-3}$ M. 2, 3, and 4 — Spectra 40 s, 12 min, and 40 min after adding ascorbate anion; [Fe^{3+}-Adr] = $2.2 \cdot 10^{-4}$ M, [AH^-] = $4.37 \cdot 10^{-3}$ M.

excess of ascorbic acid (the ratio of [AH_2]:[NBu_4OH] was about 1.05) to exclude the possible effect of alkali which also can react with anthracyclines.[8] But on the other hand, the reaction products transformed rapidly in the presence of protons giving the spectra practically identical with those of parent antibiotics. Therefore the fact that reactions with ascorbate anion did not come to completion can at least partly be explained by the presence of the excess protons.

The interaction of aclacinomycin A with O_2^- and ascorbate anion as well as of adriamycin with ascorbate anion has not been described earlier. On the basis of similarity of the spectra of parent antiobiotics and the products formed we assumed that the reactions of superoxide ion (in aprotic media and mixed solvents) and ascorbate anion (in aprotic media) with both antibiotics proceed *via* the same mechanism.



Adriamycin, R = daunosamine



Aclacinomycin A, R = aminotrisaccharide

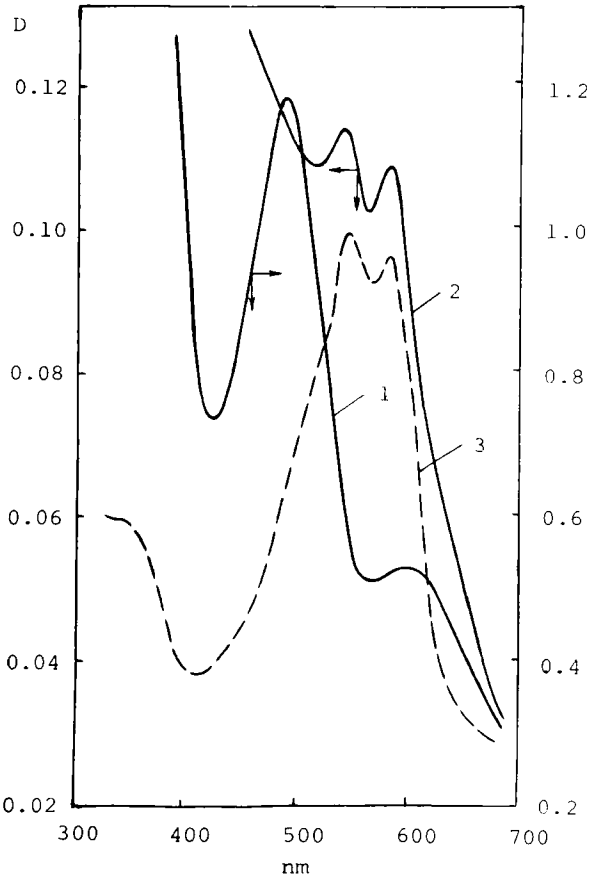


FIGURE 5 The interaction of O_2^- with Fe^{3+}-Adr in acetonitrile containing 25% of water. 1. Spectrum of Fe^{3+}-Adr, [Adr] = $0.60 \cdot 10^{-3}$ M, [$FeCl_3$] = $7.0 \cdot 10^{-3}$ M. 2. Spectrum after adding superoxide ion; [Fe^{3+}-Adr] = $5.4 \cdot 10^{-5}$ M, [O_2^-] = $1.9 \cdot 10^{-3}$ M. 3. Spectrum of the product obtained in the reaction of O_2^- with Adr in the same mixed solution.

$$O_2^- \text{ or } AH^- + \text{Anth(OR)} \rightleftharpoons O_2 \text{ or } AH\cdot + \text{Anth(OR)}^- \quad (7)$$

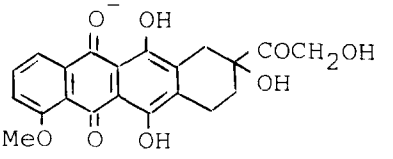
$$\text{Anth(OR)}^- \longrightarrow \text{Anth}\cdot + RO^- \quad (8)$$

$$\text{Anth}\cdot + O_2^- \text{ or } AH^- \longrightarrow \text{Anth}^- + O_2 \text{ or } AH\cdot \quad (9)$$

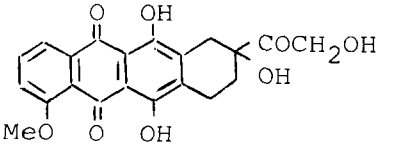
$$\text{Anth}^- + H^+ \longrightarrow \text{AnthH} \quad (10)$$

This mechanism is similar to that proposed by us earlier[8] for the interaction of O_2^- with adriamycin. It is supposed that the anthracycline semiquinone Anth(OR)$^-$ which formed during the one-electron reduction of antibiotics (reaction 7) decomposed to give the neutral deoxyaglycon semiquinone Anth· (reaction 8) which is subsequently

reduced to the $Anth^-$ anion by a second superoxide or ascorbate molecule (reaction 9).† This mechanism is principally based on the Kleyer and Koch scheme[20] for the reduction of anthracycline antibiotics by the free radical, 3,5,5-trimethyl-2-oxomorpholin-3-yl. $Anth^-$ should be the anion of the deoxyaglycon tautomer which is transformed into deoxyaglycon AnthH at acidification.[8]



$Anth^-$ (for adriamycin)



AnthH

It is known that the absorption spectrum of AnthH does not practically differ from that of parent antibiotic, that explains the "regeneration" of the initial spectrum after acidification of reaction mixture.

Thus it can be supposed that the product spectra should be related to the deoxyaglycon tautomer anions. But reaction (8) is of course impossible in the case of quinones not having the sugar moiety. Therefore the mechanism proposed cannot explain the formation of the product with a very similar spectrum in the reaction of O_2^- with quinizarin (1,4-dioxyanthraquinone).[15] So, it is possible that at the first stage of all such processes the semiquinones formed during the one-electron transfer reduction of anthraquinones form diamagnetic dimers which in the case of anthracycline antibiotics cleave to form deoxyaglycons. If it is true, then the spectra observed should be related to the dimer semiquinones.

Unlike the reactions in aprotic media, the reaction of anthracycline antibiotics with ascorbate anion in water proceeds only in the presence of Fe^{3+} ions, the process being inhibited by EDTA (Fig. 3). Therefore one-electron transfer from ascorbate anion to anthracyclines (reaction 7) in water should be catalyzed by Fe^{3+} ions. We attempted to ascertain the mechanism of this Fe^{3+}-catalysed reaction. It is well-known[22] that adriamycin forms complexes with Fe^{3+} ions in aqueous solution. We studied the interaction of ascorbate anion with Fe^{3+}-Adr in water (Fig. 4). It is evident that the reaction proceeds to form the same product as in the described above experiments with adriamycin. Therefore it seems very probable that Fe^{3+}-Adr is a true catalytic species participating in the reaction of ascorbate anion with anthracyclines in aqueous solution.

$$Fe^{3+} + Anth(OR) \rightleftharpoons Fe^{3+}\text{-}Anth(OR) \quad (11)$$

$$AH^- + Fe^{3+}\text{-}Anth(OR) \longrightarrow AH\cdot + Fe^{2+}\text{-}Anth(OR) \quad (12)$$

$$Fe^{2+}\text{-}Anth(OR) \rightleftharpoons Fe^{3+}\text{-}Anth(OR)^- \longrightarrow Anth\cdot + RO^- + Fe^{3+} \quad (13)$$

Although no change was observed in the spectrum of aclacinomycin A at the addition of $FeCl_3$ to its aqueous solution, a new maximum at 476 nm was seen in the

† It should be noted that Land *et al.*[21] observed recently the transformation of the adriamycin semiquinone into a tautomer of deoxyaglycon.

differential spectrum. Therefore we believe that aclacinomycin A also forms a complex with Fe^{3+} ions, and therefore the reaction of ascorbate anion with aclacinomycin A proceeds in aqueous solution *via* the same mechanism.

An analogous process apparently occurs during the interaction of Fe^{3+}-adriamycin with superoxide ion in aprotic media (Fig. 5). But it is very interesting that the reaction of ascorbate anion with Fe^{3+}-Adr in acetonitrile leads to the formation of a new product (λ_{max} = 486 nm). It is tempting to suppose that it may be a Fe^{2+}-Adr complex which may be more stable in aprotic media than in aqueous solution. But the difference in the reactions of Fe^{3+}-adriamycin with O_2^- and ascorbate anion in acetonitrile remains unexplained.

In conclusion we should like to comment upon the distinction between previous data and our results obtained in aqueous solution. It is obvious that the shift of equilibrium 1 to the left or equilibrium 7 to the right against the reduction potential difference is explained by the decomposition of the antibiotic semiquinone formed (reaction 8). Svingen and Powis[5] and Land *et al.*[6] have studied equilibrium 1 by a pulse radiolysis method, and therefore they determined the rate and equilibrium contants before the decomposition of antibiotic semiquinone started. It is possible that such a situation can take place in mitochondria and microsomes, but a main pathway of O_2^- formed by anthracycline antibiotics in biological systems is apparently the reduction of dioxygen by their iron complexes.[10-13]

On the grounds of the results obtained in our model system, it may be proposed that both superoxide ion and ascorbate can reduce anthracycline antibiotics in cells. Such a process can be of a significant importance to both anticancer effect and cardiotoxicity of these antiobiotics. For example it has been recently shown[14] that the reduction of adriamycin to deoxyaglycon in cells by a radical dimer (decomposed to form 3,5,5-trimethyl-2-oxomorpholin-3-yl) diminishes toxicity and improves therapeutic index of adriamycin in tumor bearing mice. But our results indicate that superoxide and ascorbate should manifest an analogous effect. Therefore the difference in a balance between superoxide ion + ascorbate and anthracycline antibiotics in normal and tumor cells may be of an importance for the difference in activity of anthracyclines in these cells.

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