erization occurred. Chromatography was performed on a Waters μ-Porasil column 7.8 mm i.d. × 30 cm (dichloromethane/light petroleum 13:7). The amount of each atropisomer was determined using a Waters Model 450 UV detector (operating at λ 420 nm) and a Hewlett-Packard 3380A integrator recorder.

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Registry No. 1, 29114-93-0; **2**, 105762-41-2; αααα-**3**, 105814-96-8; ααββ-**3**, 105728-93-6; αββα-**3**, 105814-99-1; αβαβ-**3**, 105816-42-0; αααα-**4**, 105816-41-9; ααββ-**4**, 105814-97-9; αββα-**4**, 105816-45-3; αβαβ-**4**, 105728-91-4; αααα-**5**, 105816-44-2; ααββ-**5**, 105814-98-0; αββα-**5**, 105816-43-1; αβαβ-**5**, 105728-92-5; **6**, 77287-58-2; **7**, 105728-90-3; **8**, 2338-54-7; **9**, 91319-42-5; *m*-methoxybenzaldehyde, 591-31-1; 2-amino-5-methoxytoluene, 102-50-1; 2-methyl-4-methoxybenzenediazonium, 105728-89-0; hexafluorophosphate pyrrole, 109-97-7; 2-chloro-5-methoxybenzaldehyde, 13719-61-4; 2-bromo-5-methoxybenzaldehyde, 7507-86-0.

One-Electron Reduction of Daunomycin, Daunomycinone, and 7-Deoxydaunomycinone by the Xanthine/Xanthine Oxidase System: Detection of Semiquinone Free Radicals by Electron Spin Resonance

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Abstract: High-resolution electron spin resonance spectra of semiquinones from daunomycin and its derivatives, daunomycinone and 7-deoxydaunomycinone, have been obtained during reduction by xanthine/xanthine oxidase or dithionite in partially nonaqueous media. Hyperfine coupling constants were assigned with the help of an experiment in deuterated solvent to observe the exchangeable protons and with the help of computer routines for the automatic assignment of hyperfine coupling constants. In contrast, in buffer the ESR spectrum of the daunomycin was a single broad line and that of the 7-deoxydaunomycinone exhibited axial symmetry showing strong *g*-factor anisotropy. The differences in the ESR spectra of the daunomycin semiquinone and its derivatives and the effects of the nonaqueous solvents ethanol and dimethyl sulfoxide are discussed.

Daunomycin belongs to the widespread family of anthracycline drugs used in anticancer treatment. Because of the widespread use of daunomycin, a large number of investigations have been reported in recent years, and clinical as well as molecular aspects have been recently reviewed.[1-5] Since daunomycin is a quinone compound, it is a redox active molecule, one of its reaction pathways is the one-electron reduction to a semiquinone free radical. These radicals have been proposed to attack DNA site-specifically and produce strand breaks by producing reactive oxygen radicals such as the superoxide or the hydroxyl radical.[6,7] Additionally, oxygen-derived free radicals have been proposed to be responsible for the use-restricting cardiotoxicity of the drug, which has been attributed to low levels of defensive enzymes against oxygen damage in the heart.[8] Previously, the ESR technique of spin-trapping has been employed to detect daunomycin-dependent superoxide and hydroxyl radical formation.[9-11]

Since daunomycin is an anthraquinone derivative and, like most quinones, is easily reduced to its semiquinone free radical, direct ESR has been used to detect semiquinones of daunomycin and its derivatives.[10-14] Whereas the ESR spectra of enzymatically generated anthracycline semiquinones consist of a single, unresolved line,[10-12] those obtained from chemical reduction of anthracyclines[13-16] reveal hyperfine structure. Because of this distinct difference in ESR spectra, there exists a structural dilemma as to the true identity of the semiquinones derived from daunomycin, especially in enzymatic systems. In an attempt to resolve this, we have now undertaken a study involving enzymatic reduction of anthracyclines in solutions of buffer and dimethyl sulfoxide or ethanol.

We have chosen the xanthine/xanthine oxidase reduction system for the following reasons: (i) xanthine oxidase has previously been shown to be involved during myocardial reduction of anthracyclines,[17] and (ii) the xanthine oxidase was still active in the presence of up to 50% dimethyl sulfoxide. We now report, for the first time,

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[§] Medical College of Wisconsin.

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Table I. Hyperfine Coupling Constants (in Gauss)

carbon position	daunomycin				daunomycinone		7-deoxydaunomycinone	
	Me_2SO/H_2O	H_2O[a]	methanol[b]	$Me_2SO/^2H_2O$	Me_2SO/H_2O	$MeSO/^2H_2O$	Me_2SO/H_2O[c]	$Me_2SO/^2H_2O$
1	0.88	0.8	0.92	0.9	0.9	0.82	0.90_5	0.88
2	1.01	0.8	0.92	0.98	1.01	0.98	1.01_5	1.0
3	1.45	1.5	1.44	1.49	1.47	1.45	1.42_5	1.43
6	0.52		0.5	0.1	0.58	0.04	0.57	0.08
11	0.63		0.5	0.1	0.65	0.08	0.61_5	0.1
10	2.04	2.85	1.98	1.92	2.01	1.91_5	2.31	2.13
10	1.57	2.25	1.57	1.56	1.55	1.56	1.67	1.58_5
7	2.22	2.25	2.53	2.15	2.53	2.46	2.74	2.64
7							3.66_5	3.58
	±0.005			±0.02				

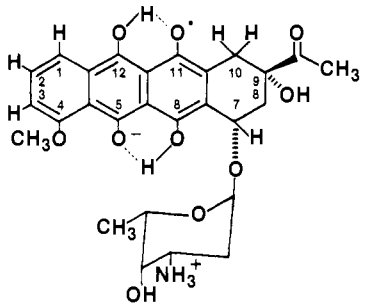
[a] Hyperfine coupling constants of the daunomycin semiquinone free radical reported by Lown and Chen.[14] [b] Hyperfine coupling constants of the daunomycin semiquinone free radical reported by Kleyer and Koch.[13] [c] For the 7-deoxydaunomycinone semiquinone free radical in Me_2SO, Kleyer and Koch[13] reported hyperfine coupling constants of 0.59 (3 H), 1.18 (1 H), 5.8 (1 H), 2.32 (1 H), and 3.06 G (1 H).

high-resolution ESR spectra of semiquinones from daunomycin and its derivatives during enzymatic reduction, and also discuss reasons for some of the existing spectral differences for anthracycline semiquinones reported here and elsewhere.[13]

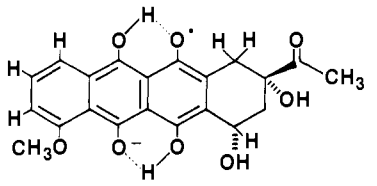
Methods

Daunomycin hydrochloride, xanthine, and xanthine oxidase were obtained from Sigma Chemical Co. and used without further purification. The daunomycinone and the 7-deoxydaunomycinone were prepared from daunomycin, and their structures were confirmed by proton NMR and mass spectrometry. The dimethyl sulfoxide (Me_2SO) was the ACS certified reagent from Fisher Scientific.

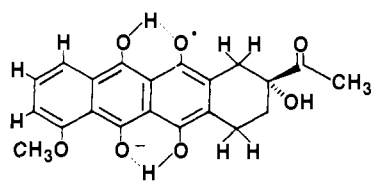
Samples for the high-resolution ESR spectra were prepared by bubbling the solutions of 0.5 mM daunomycin, daunomycinone, or 7-deoxydaunomycinone in Me_2SO (solution A) and 0.5 mM xanthine in pH 8.0



Daunomycin



Daunomycinone



7-Deoxydaunomycinone

phosphate buffer (solution B) with nitrogen for 10 min. Then 1.5-mL portions of each solution (A and B) were mixed together and again bubbled with nitrogen for 5 min. Xanthine oxidase (0.32 unit) was then added to the incubation, and the sample, without exposure to oxygen, was aspirated into an aqueous flat cell in place in the ESR cavity.[18] All experiments with high-resolution ESR spectra were done in both protonated and deuterated solvent to assign exchangeable protons.

Samples for the organic-phase concentration-dependence studies were prepared either in the above manner or by adding solid sodium dithionite (10 mM final concentration) to a solution of 0.5 mM daunomycin in pH 7.4 phosphate buffer and an appropriate volume of organic solvent which had been bubbled with nitrogen for 10 min. These samples were also aspirated into the flat cell without exposure to oxygen.

The high-resolution ESR spectra were obtained using a Varian E-109 ESR spectrometer with a quartz flat cell in an E-238 TM_{110} microwave cavity. Data acquisition was done using a Varian 936 data acquisition system and a Hewlett-Packard 9835B computer.

The other spectra were obtained using an IBM ER-200 ESR spectrometer equipped with an ES-4103 TM microwave cavity and a quartz flat cell.

Computerized assignment of hyperfine coupling constants was done in two steps;[19] potential coupling constants were found with an autocorrelation program and were then refined with an automatic tuning procedure.

Results

The addition of xanthine oxidase to an aqueous solution containing xanthine and daunomycin under nitrogen produced a broad, single-line ESR spectrum (Figure 1A). Addition of increasing concentrations of Me_2SO to the above reaction mixture caused the gradual appearance of spectral resolution (Figure 1B–D). No ESR spectra were obtained in the absence of xanthine oxidase, xanthine, or daunomycin. Having established the optimum composition of the Me_2SO–buffer solution for maximum spectral resolution of the daunomycin semiquinone spectrum (Figure 2A), we also obtained high-resolution ESR spectra during similar enzymatic reductions of daunomycinone (Figure 3A) and 7-deoxydaunomycinone (Figure 4A).

The hyperfine parameters for all three semiquinones have been determined with computer simulations (Table I). An excellent fit between the experimental (Figure 2A) and the simulated (Figure 2B) ESR spectrum of the daunomycin semiquinone was obtained. Experiments with deuterium oxide buffer (Figure 2C) show two exchangeable protons which have been previously assigned to the phenolic protons at positions 6 and 11.[13] A comparable set of spectra was obtained from daunomycinone (Figure 3), where the sugar moiety at position 7 has been replaced by an alcohol group. Figure 3A shows the experimental spectrum in protonated solution; Figure 3C shows it in deuterated solution. In Figure 3, parts B and D are the respective computer simulations with hyperfine coupling constants from Table I. The spectra of the semiquinones derived from daunomycin and daunomycinone are very similar, though not identical, which allows the assignment of the coupling constant of the proton on carbon 7 where the sugar moiety is attached. Our results as well as the molecular orbital calculations of Lown and Chen[14] indicate that the sugar moiety has no effect on the spin distribution in the daunomycin semi-

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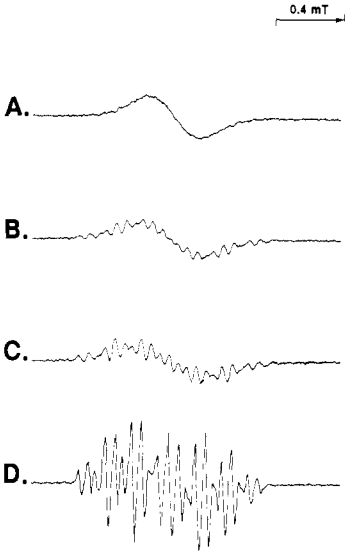

Figure 1. ESR spectra of the semiquinone free radical derived from daunomycin on reduction with xanthine/xanthine oxidase. Reaction conditions: xanthine, 0.25 mM; xanthine oxidase, 0.106 unit/mL; daunomycin, 0.25 mM; room temperature, pH 7.4. ESR conditions: microwave power, 10 mW (A–C) or 1 mW (D); modulation amplitude, 0.63 G (A–C) or 0.16 G (D); gain, 3.2×10^5; time constant, 1 s; sweep rate, 2.2 G/min. A, no dimethyl sulfoxide (Me_2SO); B, 12.5% Me_2SO; C, 25% Me_2SO; D, 50% Me_2SO.

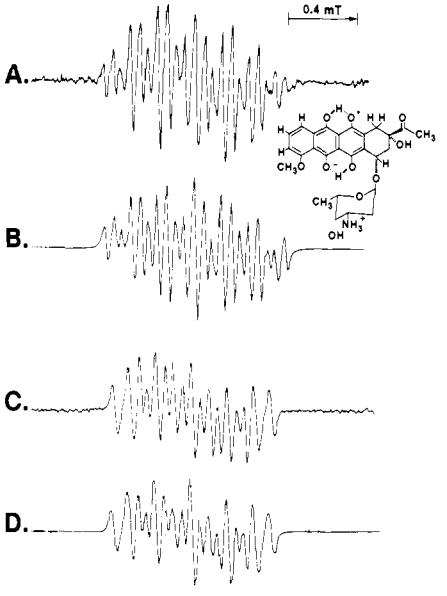

Figure 2. ESR spectra of the semiquinone free radical derived from daunomycin on reduction with xanthine/xanthine oxidase. Reaction conditions: daunomycin, 0.25 mM; xanthine, 0.25 mM; xanthine oxidase, 0.106 unit/mL; pH 8.0, 50% Me_2SO. ESR conditions: microwave power, 1 mW; modulation amplitude, 0.16 G; gain, 8×10^4 (A) or 3.2×10^4 (C); time constant, 0.25 s; sweep rate, 2.5 G/min. A, reaction in protonated solutions; B, computer simulation (hyperfine coupling constants in Table I); C, reaction in deuterated solutions; D, computer simulation.

quinone. The next set of spectra (Figure 4) was obtained from 7-deoxydaunomycinone, where the alcohol group has been reduced to hydrogen. Again, Figure 4A shows the ESR spectrum of the semiquinone free radical in a Me_2SO/buffer solution, and Figure 4C shows the ESR spectrum from an otherwise identical solution made with deuterium oxide buffer. In Figure 4, parts B and D are the corresponding computer simulations. The five small hyperfine coupling constants remain unchanged, whereas the large ones change significantly. Clearly a new coupling constant is observed and must be due to the new proton on carbon 7 where the sugar moiety has been reductively cleaved leaving a hydrogen.

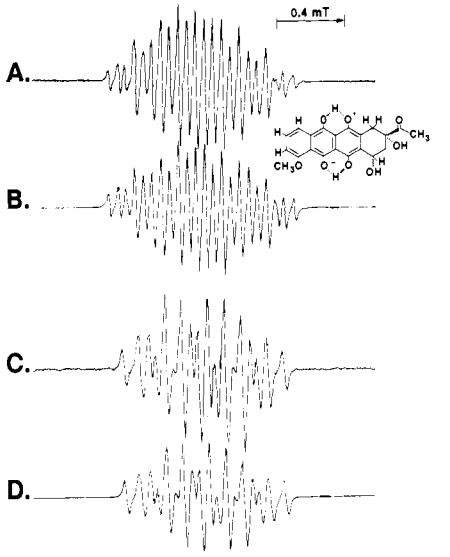

Figure 3. ESR spectra of the semiquinone free radical derived from daunomycinone on reduction with xanthine/xanthine oxidase. Reaction conditions: daunomycinone, 0.25 mM; xanthine, 0.25 mM; xanthine oxidase, 0.106 unit/mL; pH 8.0, 50% Me_2SO. ESR conditions: microwave power, 1 mW; modulation amplitude, 0.16 G; gain 1×10^4 (A) or 2×10^4 (C); time constant, 0.128 s (A) or 0.250 s (C); sweep rate, 5 G/min. A, reaction in protonated solutions; B, computer simulation (hyperfine coupling constants in Table I); C, reaction in deuterated solutions; D, computer simulation.

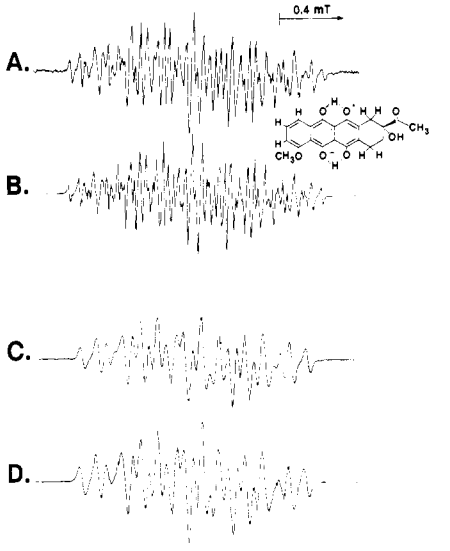

Figure 4. ESR spectra of the semiquinone free radical derived from 7-deoxydaunomycinone on reduction with xanthine/xanthine oxidase. Reaction conditions: 7-deoxydaunomycinone, 0.25 mM; xanthine, 0.25 mM; xanthine oxidase, 0.11 unit/mL; pH 8.0, 50% Me_2SO. ESR conditions: microwave power, 1 mW; modulation amplitude, 0.16 G (A) or 0.066 G (C); gain, 2×10^4 (A) or 8×10^4 (C); time constant, 0.5 s (A) or 0.25 s (C); sweep rate, 2.5 G/min (A) or 5 G/min (C). A, reaction in protonated solvents; B, computer simulation (hyperfine coupling constants in Table I); C, reaction in deuterated solvents; D, computer simulation.

The one-line ESR spectrum of daunomycin semiquinone in buffer previously has been shown to change with time into an asymmetric signal.[11] This has been attributed to formation of a semiquinone metabolite of the 7-deoxydaunomycinone or polymerization products. We now provide experimental evidence that the asymmetric ESR spectrum is indeed that of a semiquinone from 7-deoxydaunomycinone. The ESR spectrum obtained after reduction of daunomycin by dithionite possesses asymmetric features in aqueous media (Figure 5A). With increasing concentration of ethanol, which solubilizes the 7-deoxydaunomycinone, the gradual appearance of hyperfine structure is found (Figure

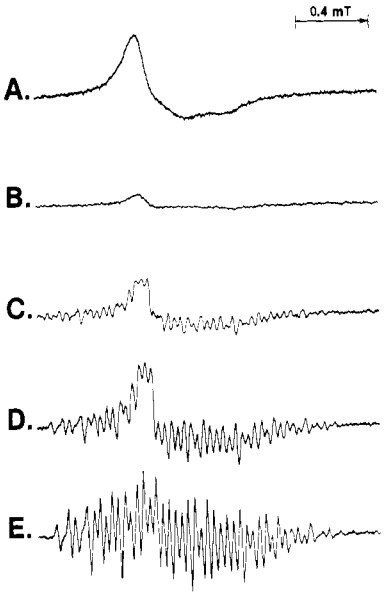

Figure 5. ESR spectra of the semiquinone free radical derived from 7-deoxydaunomycinone on reduction of daunomycin with sodium dithionite. Reaction conditions: daunomycin 0.5 mM; sodium dithionite, 1 mM, pH 7.4. ESR conditions: microwave power, 10 mW (A, B) or 1 mW (C–E); modulation amplitude, 0.63 G (A, B) or 0.16 G (C–E); gain, 4.0×10^5 (A), 1.25×10^5 (B), 6.3×10^5 (C–E); time constant, 0.5 s (A), 1 s (B–E); sweep rate, 2.2 G/min. A, no ethanol; B, 10% ethanol; C, 20% ethanol; D, 25% ethanol; E, 30% ethanol.

5B–E). Aside from solvent effects, the ESR spectrum detected after reduction of daunomycin with dithionite (Figure 5E) is identical with that formed from 7-deoxydaunomycinone (Figure 4A).

Discussion

Because of the several functional groups and the possible cleavage of the link between the tetracycline ring system and the amino sugar, the metabolism of daunomycin is extremely complex. Nevertheless, one-electron reduction of daunomycin leads to its semiquinone, which, in aqueous solution, disproportionates. Comproportionation, i.e., the back reaction of the quinone and hydroquinone, probably leads to the steady-state concentrations of the radical in aqueous medium. One- or two-electron reduction of the daunomycin leads to reductive cleavage of the amino sugar yielding 7-deoxydaunomycinone,[13] which has been reported not to intercalate into DNA in vitro and not to be antitumorogenic in cell culture.[4]

Our experiments show that all three compounds investigated, daunomycin, daunomycinone, and the cleavage product 7-deoxydaunomycinone, can undergo one-electron reduction to their respective semiquinones. This reaction was catalyzed by xanthine oxidase, an enzyme which has been proposed to be an in vivo catalyst for reductive activation in heart cytosol.[17] Our assignment of the hyperfine coupling constants of the ESR spectrum of the daunomycin semiquinone free radical generally agrees with the assignment given by Lown and Chen[14] and by Kleyer and Koch.[13] Our experiments in deuterated solutions confirm the assignment of the hyperfine coupling constants of the phenolic protons at positions 6 and 11 given by Kleyer and Koch.[13] Observation of phenolic protons with hydrogen bonding to the quinone oxygen has also been reported by Freed and Fraenkel[20] in the case of naphthazarin. With 7-deoxydaunomycinone, however, there is a considerable discrepancy between our spectrum and that of Kleyer and Koch[13] as well as in the assigned hyperfine coupling constants. The reasons for the observed discrepancy are not clear.

It should be pointed out that we present here the first high-resolution ESR spectrum of a semiquinone free radical derived from 7-deoxydaunomycinone. In earlier studies the asymmetric singlet seen in Figure 5A had been assigned to the 7-deoxydaunomycinone based on kinetic considerations rather than on spectroscopic information. Our study shows clearly the connection between the singlet and the high-resolution ESR spectrum from 7-deoxydaunomycinone as well as the consistency with the other two semiquinones derived from daunomycin and daunomycinone.

For the first time we showed that the transition of daunomycin and its derivatives from low-resolution ESR spectra to high-resolution ESR spectra and their strong dependence on organic solvent is probably due to the solubility of the respective free radicals. The self-association of daunomycin itself into dimers is well-known.[21,22] The appearance of an ESR spectrum with *g*-factor anisotropy for 7-deoxydaunomycinone in buffer could be an effect of its hydrophobicity leading to large molecular weight structures, possibly aggregates of stacked molecules. In any case, in buffer the semiquinone of 7-deoxydaunomycinone is immobilized on the ESR time scale.

The structure of the daunomycin, daunomycinone, and 7-deoxydaunomycinone semiquinones is now known to be inherently the same in both chemical and enzymatic systems, with previously described differences shown to be solvent effects. We are now in a position to apply ESR to the study of these radicals in a variety of more complicated systems such as their interactions with DNA.[11,15,16,23] We also feel that the present mode of quinone reduction by xanthine and xanthine oxidase in Me_2SO-buffer solutions is quite general and can be applied to other biologically relevant quinones with limited water solubility.

Acknowledgment. This work has been supported by the National Institutes of Health Grants GM-29035 and RR-01008.

Registry No. Ethanol, 64-17-5; dimethyl sulfoxide, 67-68-5; daunomycin, 20830-81-3; xanthine oxidase, 9002-17-9; daunomycinone, 21794-55-8; 7-deoxydaunomycinone, 32384-98-8; daunomycin semiquinone, 73610-99-8; daunomycinone semiquinone, 91740-61-3; 7-deoxydaunomycinone, 95976-56-0.

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