

# REDUCTION OF ANTITUMOUR MITOSENS IN NON-AQUEOUS AND AQUEOUS ENVIRONMENT. AN ELECTRON SPIN RESONANCE AND CYCLIC VOLTAMMETRY STUDY

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Chemical reduction of mitosenes under aerobic conditions in DMSO showed characteristic ESR signals of the mitosene derived semiquinone free radicals. However, these signals diminished strongly upon addition of water to the reaction mixture, indicating a short lifetime of the mitosene semiquinone free radicals under aqueous conditions. In addition, enzymatic one-electron reduction of these mitosenes with either xanthine oxidase or purified NADPH cytochrome P450 reductase under anaerobic conditions showed no signals of the mitosene semiquinone free radicals. Subsequent cyclic voltammetry measurements demonstrated facilitation of the further one-electron reduction of the mitosene semiquinone free radicals in the presence of water in comparison with non-aqueous conditions. The present results strongly suggest that in the presence of water relatively stable hydroquinones are formed upon reduction of mitosenes. Consequently, the steady state concentrations of mitosene semiquinone free radicals will be lowered substantially in aqueous environment. Thus under physiological conditions, two-electron reduction and formation of the mitosene hydroquinone might be important in processes leading to DNA alkylation by these mitosenes.

**KEY WORDS:** Mitosenes, Mitomycin C, Semiquinone free radical, reductive activation, hydroquinone.

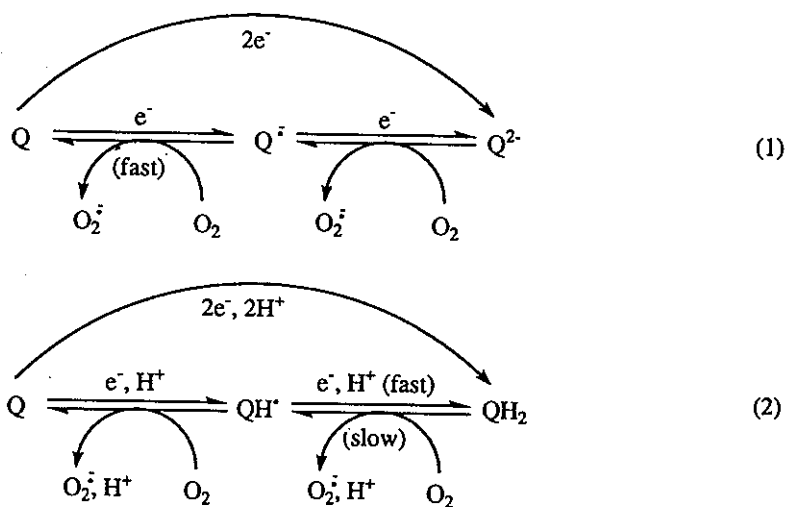
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## INTRODUCTION

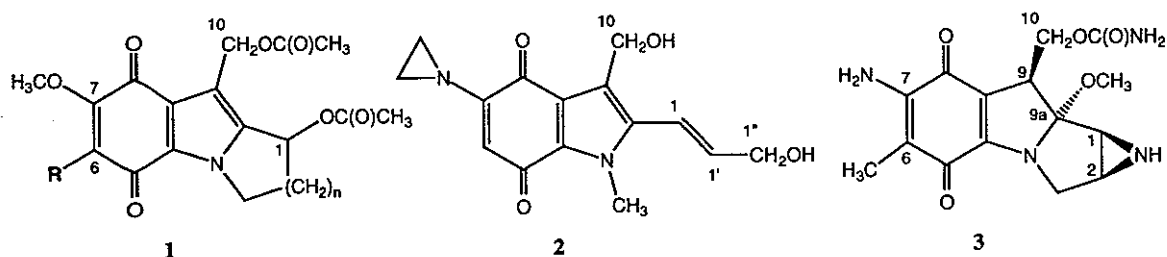
Mitosenes (basic structure 1) are potentially cytostatic quinones, structurally related to E09 (structure 2), which is currently undergoing Phase I clinical trial,<sup>1</sup> and to the clinically relevant antitumour agent mitomycin C (structure 3). In order to exert its cytostatic effect, mitomycin C first has to be activated bioreductively. Reduction of mitomycin C generates a mitosene intermediate with electrophilic centers at the C-1 and C-10 sites.<sup>2</sup> Reductive activation of mitomycin C in the presence of DNA has been shown to result in DNA adduct formation,<sup>3-5</sup> of which most likely the interstrand DNA cross-links constitute the major molecular cause for its antitumour activity.<sup>6</sup> As a mitosene structure is an intermediate in the reductive activation process of mitomycin C, a series of structurally related mitosenes have been synthesized and their antitumour activity was evaluated in *in vitro* tumour models.<sup>7,8</sup> Similar to mitomycin C, mitosenes are activated bioreductively.<sup>9</sup> Moreover, it has been demonstrated that mitosenes can also induce interstrand cross-linking of DNA.<sup>9</sup>

Mitosenes can be reduced either via a one- or a two-electron reduction mechanism, resulting in the formation of the semiquinone free radicals and dianions of the mitosenes, respectively. In aqueous environment, under physiological conditions, the dianions are protonated to form the corresponding hydroquinones.<sup>10</sup> The involved reactions are shown in scheme 1 by equations (1) and (2), respectively. However, the precise molecular mechanisms underlying these reduction reactions



are not fully understood. Moreover, it is not known whether the respective semiquinone free radicals or the hydroquinones of the mitosenes are involved in processes leading to antitumour activity. In the case of mitomycin C, it has been suggested that one-electron reduction to a semiquinone free radical is sufficient for bioactivation and DNA alkylation.<sup>11</sup> However, this one-electron reduction reaction is particularly sensitive to molecular oxygen due to reoxidation of the semiquinone free radicals under concomitant formation of superoxide anion radicals, a process generally referred to as redox-cycling.<sup>12</sup> The enzymatic (via superoxide dismutase) or spontaneous dismutation of superoxide anion radicals can produce hydrogen peroxide, and in the presence of trace amounts of heavy metals even more deleterious hydroxyl radicals are formed. The formation of reactive hydroxyl radicals can cause membrane lipid peroxidation, protein-, and DNA-damage and also affect cell replication.<sup>13</sup>

In order to elucidate the molecular mechanisms underlying the bioreductive activation of mitosenes, we decided to study this process by both electron spin resonance (ESR) and cyclic voltammetry (CV) techniques. For this purpose a series of six mitosenes (see structure 1 and Table I) was used, varying in size of ring C (5- or 6-membered), and the substituent attached to the C-6 site.



## MATERIALS AND METHODS

Mitosenes were synthesized as described previously.<sup>7</sup> *N,N*-dimethylformamide (DMF) p.a. and dimethylsulfoxide (DMSO) p.a. were purchased from Baker (Deventer, The Netherlands). DMSO used for electrochemical experiments was stored on molecular sieves. Tetrabutylammoniumperchlorate (TBAP) (electrochemical grade) was obtained from Fluka Chemie (Buchs, Switzerland). Phenobarbital (PB) was from Brocacef (Maarsse, The Netherlands). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADH, NADPH and xanthine oxidase were purchased from Boehringer (Mannheim, Germany). Diethylenetriamine-pentaacetic acid (DTPA) was from Janssen Chimica (Beerse, Belgium). NADPH cytochrome P450 reductase and cytochrome P450 2B1 were purified from livers of PB-induced rat livers, as described previously.<sup>14,15</sup>

**Measurement of mitosene semiquinone free radicals by ESR** For reference purpose,  $\text{NaBH}_4$ -mediated semiquinone free radical formation from mitosenes was determined with ESR by adding 40 mM  $\text{NaBH}_4$  to 4 mM mitosene in 100% DMSO under aerobic conditions. ESR measurements were performed with a Bruker ESP-350 spectrometer/ESP 1600 data processor. Reactions were carried out at room temperature and the spectra were recorded immediately after the initiation of the reaction in an ESR quartz flat cell mounted in a cavity with a nominal microwave power of 20 mW and microwave frequency of 9.78 GHz. Incubation conditions and ESR parameters are indicated in the respective figure legends.

**Cyclic voltammetry measurements** Cyclic voltammetry (CV) measurements were performed at room temperature with an electrochemical cell consisting of an EG&G 303 mercury working electrode, an Ag/AgCl (saturated KCl) reference electrode and a platinum wire auxiliary electrode. A computer controlled potentiostat (Autolab, Eco Chemie B.V., Utrecht, The Netherlands) with the GPES 2.2 software package was used. DMSO was dried on molecular sieves before use, and was supplemented with 0.075 M TBAP as supporting electrolyte. Cyclic voltammograms of 0.8 mM mitosene solutions were recorded under anaerobic conditions after purging the reaction mixture with nitrogen for 10 min. Nitrogen used throughout these electrochemical experiments was passed through a methylviologen/proflavine photochemical system (modified from ref. 16) in order to remove traces of oxygen and finally through a calcium chloride drying tube to remove traces of water.

Reduction/oxidation cycles were recorded between  $-0.3$  and  $-1.8$  V vs Ag/AgCl at scan-rates of 20, 100 and 500 mV/s. Moreover, for mitosenes 3, 4, 7, and 8, cyclic voltammograms were recorded between  $-0.3$  and  $-1.1$  V.

The influence of water on reduction characteristics of mitomycin C and mitosenes was tested by titrating the reaction mixture with various amounts of 0.1 M phosphate buffer, pH 7.4. The peak potential of the first ( $E_{pc1}$ ) and the second cathodic wave ( $E_{pc2}$ ) was used as indicator for the first and the second one-electron reduction potential, respectively.

## RESULTS AND DISCUSSION

In order to characterize and identify the semiquinone free radicals of the mitosenes, these compounds were reduced by  $\text{NaBH}_4$  in DMSO under aerobic conditions. This method is based on the ability of  $\text{NaBH}_4$  to reduce the mitosenes in a direct two-electron reduction to the respective dianions. In the presence of molecular oxygen, the dianions are oxidized to the semiquinone free radicals which can be detected by ESR under steady state conditions. As shown in Figure 1, chemical reduction of the mitosenes in 100% DMSO yielded characteristic ESR signals of the mitosene semiquinone free radicals. The  $g$ -values for these mitosenes that were calculated from the respective ESR spectra are shown in Table I. Under anaerobic conditions no ESR signals of the mitosene semiquinone free radicals were detected. Moreover, when either  $\text{NaBH}_4$  or mitosenes were omitted, an ESR signal could no longer be detected.

Inasmuch as strong ESR signals of the semiquinone free radicals of the mitosenes were observed, it was considered of interest to investigate the enzymatic one-electron reduction of these compounds. Enzymes known to catalyze one-electron reduction of quinones are xanthine oxidase and NADPH-cytochrome P450 reductase.<sup>17</sup> Xanthine oxidase mediated mitosene semiquinone free radical formation was determined with ESR at room temperature by adding 0.04 U/mL xanthine oxidase to 5 mM mitosene and 10 mM NADH in 50 mM phosphate buffer, pH 7.8, as described previously by Goepfert *et al.*<sup>17,18</sup> Mitosenes were dissolved in DMSO so that the final DMSO concentration was less than 1% (v/v). It has been shown previously that this concentration of DMSO does not affect xanthine oxidase activity.<sup>17</sup> HPLC analysis has shown that reduction of mitosenes by xanthine oxidase results in the conversion of the compounds at the C-1 and C-10 position, indicating that these mitosenes are

TABLE I  
Structures of mitosene compounds. Also included are  $g$ -values of the mitosene-derived semiquinone free radicals in 100% DMSO. Incubation conditions and ESR parameters are indicated in the legend to Figure 1

Compound	Code	n	R	$g$ -value
3	WV2	2	-H	2.00886
4	WV7	1	-H	2.00887
5	WV3	2	-Br	2.00815
6	WV10	1	-Br	2.00815
7	WV12	2	-CH <sub>3</sub>	2.00810
8	WV15	1	-CH <sub>3</sub>	2.00815

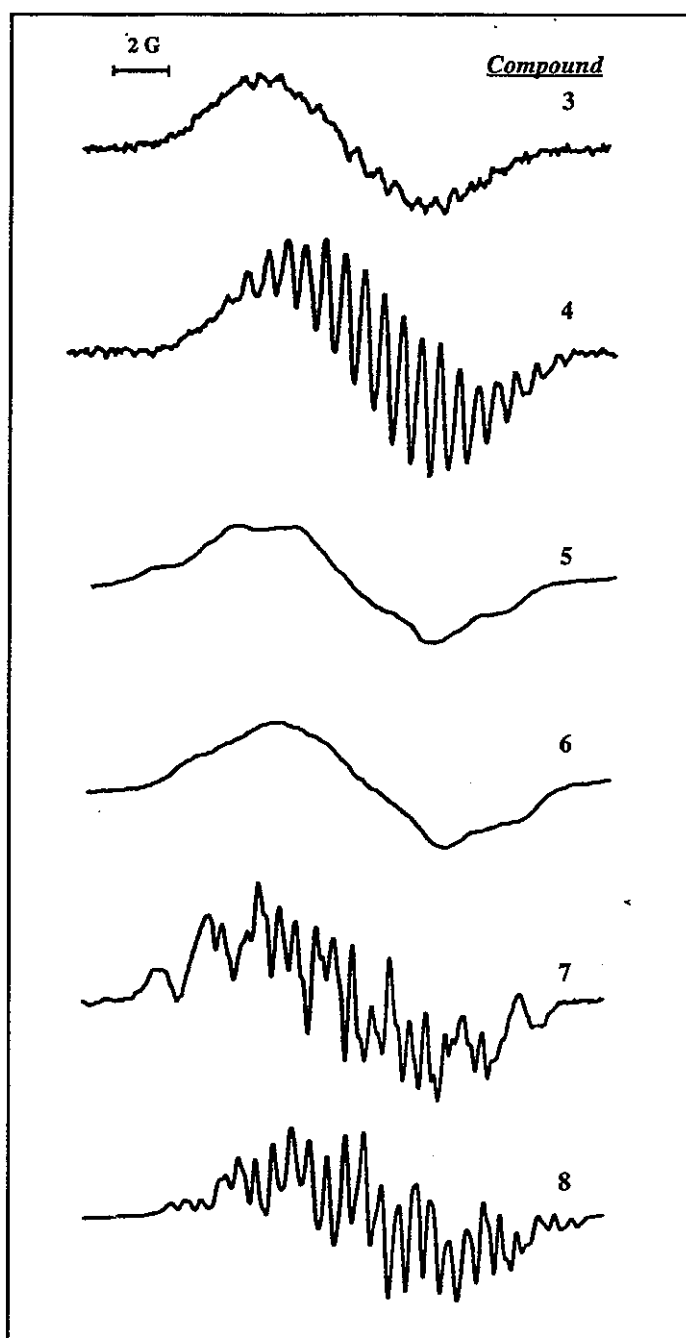


FIGURE 1 ESR spectra of the mitosene-derived semiquinone free radicals. ESR spectra were recorded upon aerobic incubation of 4 mM mitosene (compounds 3-8) with 40 mM  $\text{NaBH}_4$  in 100% DMSO. The ESR settings were as follows: microwave power, 20 mW; microwave frequency, 9.78 GHz; midfield set at 3480 G; time constant, 0.7 s; modulation frequency, 100 kHz; modulation amplitude, 0.1 G; number of scans, 5. Representative experiments out of at least four are shown.

substrates for this enzyme (manuscript in preparation). Unfortunately, no ESR signals could be detected in the incubation mixtures under anaerobic conditions. Similarly, anaerobic incubations of 5 mM mitosene in the presence of rat liver microsomes (1 mg protein/mL) and a NADPH-regenerating system consisting of 1 mM NADPH, 5 mM glucose-6-phosphate, and glucose-6-phosphate dehydrogenase (1 U/mL) in 50 mM phosphate buffer (pH 7.4, containing 0.5 mM DTPA)

showed no ESR signals of the mitosene semiquinone at all. In addition, anaerobic incubation of mitosenes (5 mM) with purified NADPH-cytochrome P450 reductase (70 nM) and a NADPH-regenerating system also failed to produce detectable ESR-signals of mitosene semiquinone free radicals. Finally, incubations of mitosenes (5 mM) with a fully reconstituted system of purified cytochrome P450 2B1 (70 nM), NADPH-cytochrome P450 reductase (70 nM) and NADPH (1 mM) yielded no ESR signals at all. It should be noted that a fully reconstituted system of purified cytochrome P450 2B1 and NADPH-cytochrome P450 reductase has been shown to enhance the one-electron reduction of quinones under anaerobic conditions.<sup>17-19</sup>

Previously, similar observations have been reported for mitomycin C. Chemical reduction of an aqueous solution of mitomycin C by  $\text{NaBH}_4$  resulted in an intermediate species with a lifetime of approximately 10 seconds.<sup>20</sup> Moreover, it has been shown that reduction of mitomycin C in rat hepatic microsomal incubations with NADPH under anaerobic conditions produces an ESR signal with a short lifetime of approximately 4 min.<sup>21</sup> Thus the mitomycin C semiquinone free radical is rather unstable in an aqueous environment under physiological conditions.

The structural similarity between mitomycin C and mitosenes probably explains why both chemical and enzymatic reduction of mitosenes in aqueous solutions failed to produce an ESR signal of the respective semiquinone free radical. Therefore, the influence of  $\text{H}_2\text{O}$  on the semiquinone free radical formation of mitosenes (4 mM) upon chemical reduction with 40 mM  $\text{NaBH}_4$  in DMSO was tested using ESR techniques. As shown in Figure 2, already in the presence of 10% v/v of  $\text{H}_2\text{O}$  in the reaction mixture the ESR signal decreased strongly in the case of mitosene 8, whereas in the presence of higher concentrations of  $\text{H}_2\text{O}$  the ESR signal of the mitosene

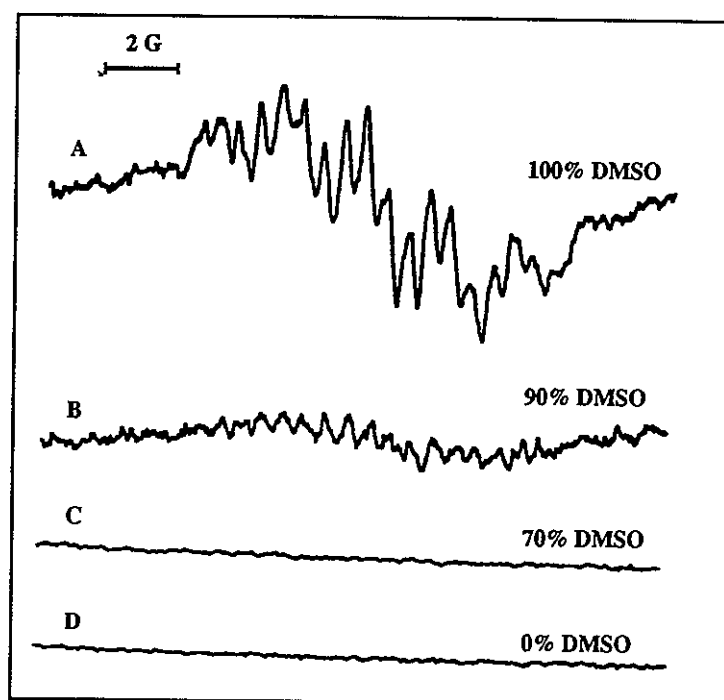


FIGURE 2 Effect of  $\text{H}_2\text{O}$  on the mitosene semiquinone free radical obtained upon chemical reduction of mitosene 8 with  $\text{NaBH}_4$  in DMSO. ESR spectra were obtained upon aerobic incubation of 4 mM mitosene 8 in 100% DMSO (spectrum A) or in the presence of 10%, 30% or 100%  $\text{H}_2\text{O}$  (spectra B, C, and D, resp.), as described in Materials and Methods. ESR settings were as described in the legend to Figure 1. Representative experiments out of four are shown.

semiquinone free radical disappeared completely. Similar observations were made for the other 5 mitosenes tested (data not shown).

The apparent lower steady state concentration of mitosene semiquinone free radicals upon chemical reduction in the presence of H<sub>2</sub>O may be explained by the protonation of the respective dianion to the resulting hydroquinone. One may argue that, due to the high stability of the hydroquinone form,<sup>10</sup> this intermediate is reoxidized slowly, resulting in low steady-state concentrations of the respective semiquinone free radical. The absence of ESR-detectable mitosene semiquinone free radicals upon one-electron reduction using either xanthine oxidase or NADPH cytochrome P450 reductase also suggests, in analogy with mitomycin C<sup>21</sup>, a very short lifetime of the mitosene semiquinone free radicals.

In order to get more insight in the reduction process of mitosenes under aqueous and non-aqueous conditions, CV measurements were performed. Generally, the equilibrium: quinone + hydroquinone  $\rightleftharpoons$  2 semiquinone free radicals depends on solvent and pH because of ionization of the hydroquinone and the semiquinone free radicals. (For pH-dependence of quinone reduction potentials, see ref 22). Under non-aqueous conditions (e.g. in DMSO), protonation of semiquinone free radicals and dianions does not occur. CV scans in DMSO/0.075 M TBAP, recorded at a scan-rate of 20 mV/s, are shown in Figure 3. The cathodic wave peak potentials ( $E_{pc}$ ) and the anodic wave peak potentials ( $E_{pa}$ ) observed for the mitosenes are summarized in Table II. In DMSO/0.075 TBAP two separate reduction steps were detected, the first step representing the first one-electron transfer to form the mitosene semiquinone free radical, whereas the second step represents the further one-electron reduction to the mitosene dianion. Bromine substitution at the C-6 position of mitosenes 5 and 6 resulted in less negative  $E_p$  values, due to the electron-withdrawing character of this substituent. In the CV scans of the brominated mitosenes 5 and 6, two additional reduction waves (denoted 3 and 4 in Figure 3) were observed. The height of these peaks did not increase upon multicycle scanning of the samples (data not shown). Taking the  $E_{pc}$  values of the bromine substituted mitosene into consideration, these reduction waves 3 and 4 probably represent the first and second one-electron reduction steps of some debrominated mitosene that was originally present in the samples.

An excellent correlation was obtained between the  $E_{pc1}$  values, as measured in this study, and the previously measured half-wave reduction potential ( $E_{1/2}$ ) of these compounds in 0.1 M phosphate buffer pH 7.4<sup>8</sup>:

$$\begin{aligned} E_{pc1} &= 12.2 + 2.59 E_{1/2} \\ n &= 5, r^2 = 0.997, s = 6.6, F = 1054.8 \end{aligned} \quad (3)$$

In order to investigate the influence of H<sub>2</sub>O on the reduction characteristics of mitosenes, cyclic voltammograms were recorded of 80 mM mitosene solutions in DMSO/0.075 M TBAP, in the presence of increasing amounts of 0.1 M phosphate buffer, pH 7.4. The  $E_{pc}$  and  $E_{pa}$  values of a typical experiment using mitosene 7 are summarized in Table III, and some representative cyclic voltammograms are shown in Figure 4. For the other mitosenes, similar results were obtained (data not shown). Upon titration with 0.1 M phosphate buffer at pH 7.4, the peak potentials of both one-electron reductions shifted to less negative values. However, the second one-electron reduction step was affected more dramatically than the first one. These results indicate the facilitation of the second one-electron reduction step to form the dianion or hydroquinone form of the mitosene in the presence of increasing H<sub>2</sub>O concentrations. Interestingly, the same effect was also observed for mitomycin C (Table IV).

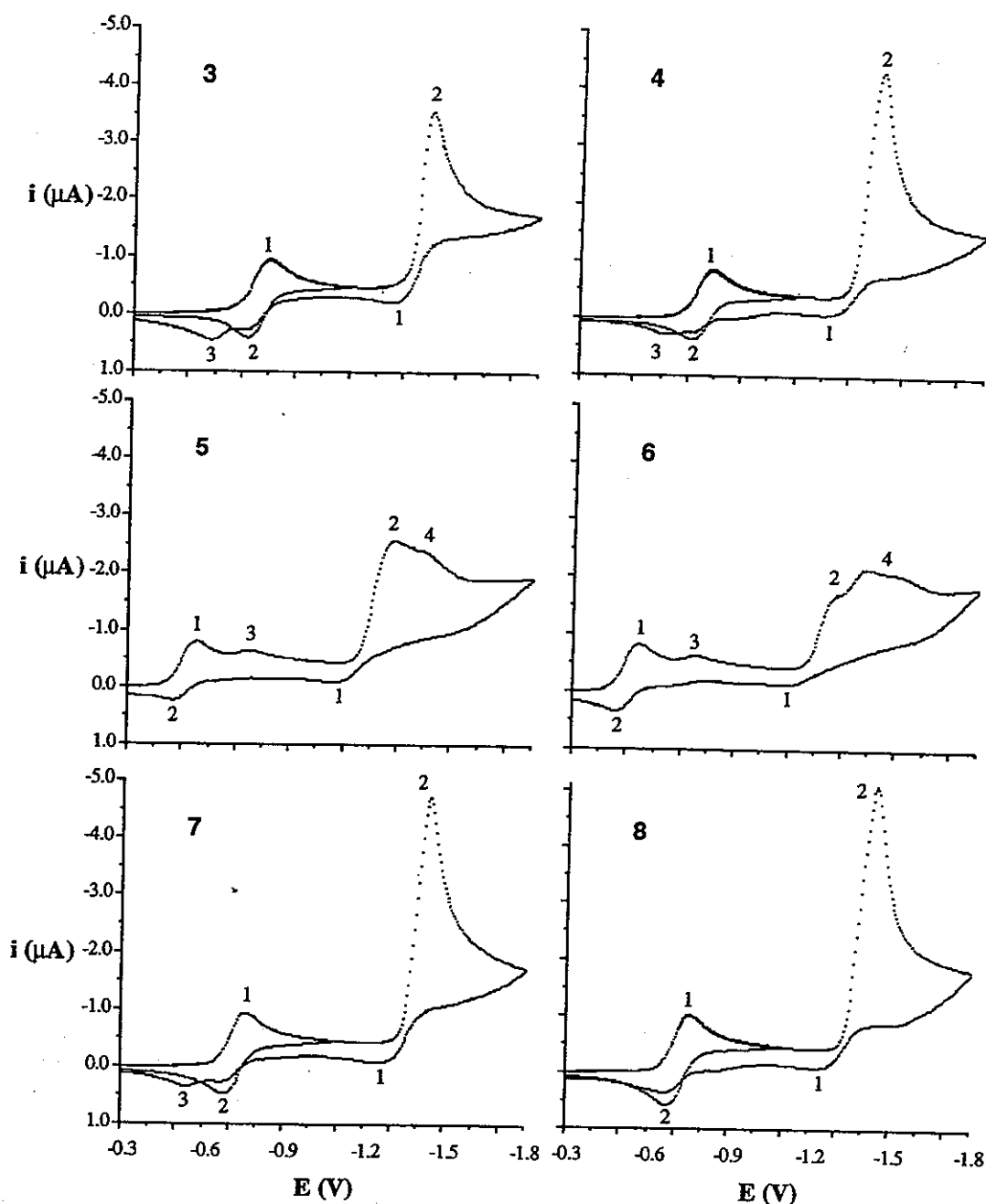


FIGURE 3 Cyclic voltammograms of mitosenes 3–8 in 100% DMSO. Voltammograms of 0.8 mM mitosene solutions were recorded under anaerobic conditions at a scan-rate of 20 mV/s as described in Materials and Methods.

As shown in Figure 5, both values of  $E_{pc1}$  and  $E_{pc2}$  for mitosene 7 decrease linearly in a  $H_2O$ -concentration dependent manner. By extrapolating the data it appears that the difference between the first and second cathodic peak potential decreases to zero in the presence of approximately 30% (v/v) of  $H_2O$ . Thus upon reduction of the mitosene in the presence of  $H_2O$ , at concentrations higher than 30% (v/v), the hydroquinone form of the mitosene may be formed almost instantaneously (Scheme 1, eq. 2). This observation is in agreement with thermodynamic data obtained from electrochemical experiments and theoretical calculations using



TABLE II

Cyclic voltammetry cathodic peak potentials ( $E_{pc}$ ) and anodic peak potentials ( $E_{pa}$ ) in DMSO/0.075 M TBAP, and  $E_{1/2}$  values of mitosene compounds in 0.1 M phosphate buffer pH 7.4

Compound	$E_{pc1}$	$E_{pc2}$	$E_{pc3}$	$E_{pc4}$	$E_{pa1}$	$E_{pa2}$	$E_{pa3}$	$E_{1/2}^*$
	(mV vs Ag/AgCl)							
3	-803	-1469			-1267	-725	-588	-314
4	-794	-1471			-1223	-715	-618	-311
5	-554	-1292	-754	-1379	-1072	-466		n.d. <sup>†</sup>
6	-544	-1295	-754	-1492	-1091	-461		-215
7	-764	-1498			-1272	-681	-540	-303
8	-759	-1505			-1238	-676		-295

\* ref 8

† n.d., not determined

TABLE III

Cyclic voltammetry cathodic peak potentials ( $E_{pc}$ ) of 0.8 mM mitosene 7 in DMSO/0.075 M TBAP and various amounts of H<sub>2</sub>O (as 0.1 M phosphate buffer, pH 7.4) at a scan-rate of 100 mV/s

% H <sub>2</sub> O	$E_{pc1}$	$E_{pc2}$
	(mV vs Ag/AgCl)	
0	-759	-1487
0.95	-754	-1433
2.8	-740	-1389
5.5	-725	-1340
8.8	-715	-1267
16.1	-686	-1013
22.4	-667	-872

substituted 1,4-benzoquinones, showing that protonation of the dianions of these 1,4-benzoquinones to the hydroquinones results in a negative free energy change, and therefore facilitates the transfer of the second electron to the semiquinone free radical.<sup>23</sup> Similarly, it is expected that the second one-electron reduction of the unprotonated mitosene semiquinone free radical to the dianion (Scheme 1, eq. 1) will be energetically less favourable than the reduction of the protonated mitosene semiquinone free radical to the corresponding hydroquinone (Scheme 1, eq. 2).

According to the present findings, chemical reduction of mitosenes under physiological conditions will primarily result in the formation of mitosene hydroquinones. Due to the high stability of mitosene hydroquinones when compared to mitosene semiquinone free radicals, the hydroquinone form may reach its target over a longer distance, and consequently may play an important role in DNA cross-linking that has been demonstrated previously upon electrochemical reduction of mitosenes in the presence of calf thymus DNA.<sup>9</sup> However, although the lifetime of the mitosene semiquinone free radical may be very short, a role for mitosene semiquinone free radicals in DNA alkylation can not be excluded. Preliminary experiments demonstrate the formation of H<sub>2</sub>O<sub>2</sub>, most likely due to dismutation of superoxide anion radicals, upon one-electron reductive activation of mitosenes in rat liver microsomal fractions in the presence of NADPH (manuscript in preparation).

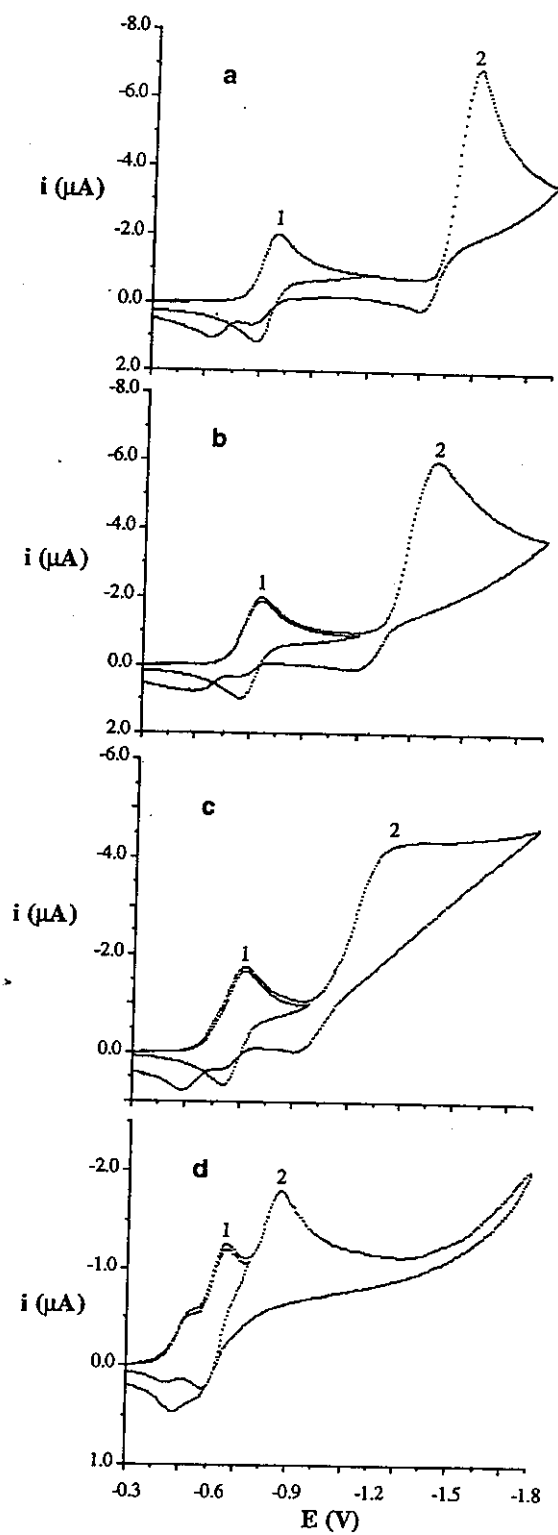


FIGURE 4 Cyclic voltammograms of a 0.8 mM solution of mitosene 7 in 100% DMSO (a), or containing 2.8% (b), 8.8% (c), or 22.4% (d) 0.1 M phosphate buffer pH 7.4 at a scan-rate of 100 mV/s. Conditions were as described in Materials and Methods.

TABLE IV

Cyclic voltammetry cathodic peak potentials ( $E_{pc}$ ) of 0.8 mM mitomycin C in DMSO/0.075 M TBAP and various amounts of  $H_2O$  (as 0.1 M phosphate buffer, pH 7.4) at a scan-rate of 100 mV/s

% $H_2O$	$E_{pc1}$ (mV vs Ag/AgCl)	$E_{pc2}$
0	-920	-1594
4.8	-886	-1433
9.1	-857	-1296
13.0	-837	-1194
16.7	-813	-1121
23.1	-784	-979

Therefore, it seems that mitosene semiquinone free radicals are likely to be formed upon enzymatic one-electron reduction. It is known that mitomycin C is reduced enzymatically in a one-electron reduction step to form the mitomycin C semiquinone free radical, which is rapidly reoxidized in the presence of molecular oxygen, under concomitant formation of superoxide anion free radicals and  $H_2O_2$ .<sup>19</sup> Due to 1) the structural similarity between mitomycin C and the mitosenes and 2) the unique reductive behaviour of these compounds, a similar mechanism in the formation of  $H_2O_2$  by mitosenes can be anticipated in subcellular fractions of the rat liver.

Concluding, the present study demonstrates that the formation of mitosene semiquinone free radicals upon chemical reduction of mitosenes in non-aqueous environment can be detected using standard ESR techniques. However, chemical or

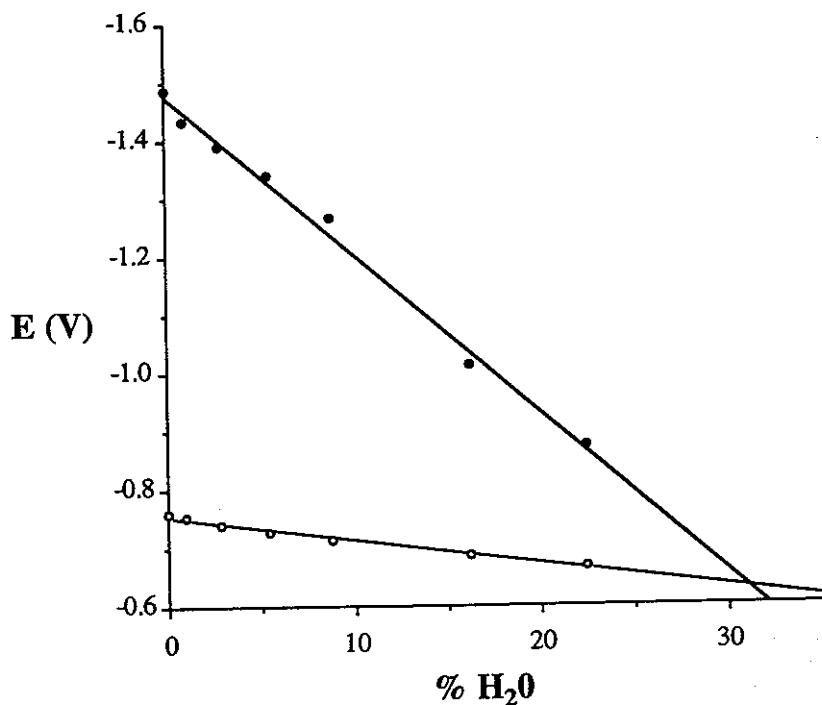


FIGURE 5 First and second one-electron reduction peak potentials ( $E_{pc1}$  (○) and  $E_{pc2}$  (●), respectively) of mitosene 7 vs concentration of  $H_2O$  (as 0.1 M phosphate buffer, pH 7.4) in DMSO measuring solution.

enzymatic reduction in aqueous environment failed to produce detectable ESR signals of the mitosene semiquinone free radicals. The relatively short lifetime of mitosene semiquinone free radicals in aqueous environment indicates that these radicals may reoxidize readily in the presence of molecular oxygen. CV measurements have demonstrated that one-electron reduction of the mitosene semiquinone free radical is facilitated under aqueous conditions in comparison with non-aqueous conditions. The hydroquinone formed in aqueous environment is relatively stable under aerobic conditions, and therefore less mitosene semiquinone free radicals will be formed due to slow reoxidation of the hydroquinone. As a consequence, the steady-state concentration of the mitosene semiquinone free radical under aqueous conditions will be substantially lower than under non-aqueous conditions. These results suggest a substantial role of the mitosene hydroquinone in DNA alkylation. In this context, the two-electron reducing enzyme DT-diaphorase has attracted attention in recent years as an activating enzyme for bioreductive cytostatic compounds, especially since its activity is high in certain tumour cells.<sup>24</sup> In fact, it appeared that DT-diaphorase may be important for mitosene bioactivation in Chinese hamster V79 cells, leading to substantial cytotoxic effects (I.J. Stratford, personal communication). Therefore the role of two-electron reductive activation of mitosenes will be further exploited.

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