Electron paramagnetic resonance and cyclic voltammetry studies of the cations and anions of α-aminoanthraquinones obtained by electrochemical oxidation/reduction

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The 1,4-diaminoanthraquinone dyes Disperse Violet 1 and Disperse Blue 14 have been studied by cyclic voltammetry and EPR spectroscopy. The cation radicals, obtained by *in situ* electrochemical one-electron oxidation, can be characterised as aromatic diamino radicals (semiquinonediimines), with a spectroscopic pattern similar to those of Wurster's Blue-type radicals. The anion radicals, obtained by one-electron reduction, are of the semiquinone type. For the cation radicals all amino protons are unequivocally identified by deuterium exchange.

The new data show that single-line spectra observed from the antitumour antibiotics mitoxantrone and ametantrone must have arisen by a process more complex than simple one-electron oxidation of the parent compounds.

Introduction

The anthracycline antitumour antibiotics doxorubicin and daunorubicin are considered to have the broadest spectrum of clinical activity and are among the most widely prescribed. Their clinical application, however, is severely limited owing to the associated cumulative dose-dependent cardiotoxicity.[1] Intensive efforts have therefore been made both to elucidate the mechanism of action of these compounds, and to develop alternative agents with comparable antitumour efficacy but diminished cardiotoxicity.

Among the more promising new agents are the aminoanthracenedione derivatives mitoxantrone (MXH_2) and ametantrone (AM). A number of recent studies have focused on biologically relevant properties of these agents including their possible redox activity and oxidative enzymic metabolism.[1-4]

Electron paramagnetic resonance (EPR) spectroscopic and photometric studies showed that mitoxantrone is subject to easy oxidative metabolism by the horseradish peroxidase system (HRP), with concomitant generation of a free radical. The radical gave rise to an EPR spectrum consisting of a single line with no hyperfine structure, a g factor of ~2.003 and a linewidth of 10–16 G.[2,3] A similar spectrum was observed after electrooxidation of mitoxantrone at a cell potential of 0.85 V.[1]

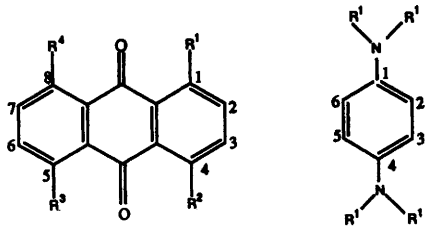
A one-electron mechanism for the HRP–H_2O_2-catalysed oxidation of mitoxantrone has been put forward[3] [eqn. (1)]. After

$$MXH_2 \overset{HRP-H_2O_2}{\rightleftharpoons} MXH_2^{\cdot+} \overset{-2H}{\longrightarrow} MH_2^{\cdot+} \overset{-e^-}{\rightleftharpoons} MH_2^{2+} \quad (1)$$

the initial one-electron oxidation of the drug (MXH_2), a cyclisation reaction furnishes a cation radical of the metabolite, termed MH_2 and shown to be a substituted hexahydronaphtho-[2,3-*f*]quinoxaline-7,12-dione.[1] The cation $MH_2^{\cdot+}$ participates in redox equilibria with the neutral MH_2 metabolite and its unstable fully oxidized form (MH_2^{2+}).

If the one-electron mechanism (the most common for HRP-catalysed oxidation reactions) is accepted, the experimental data obtained so far do not permit discrimination between $MXH_2^{\cdot+}$ and $MH_2^{\cdot+}$ radicals, although the free radical species observed is most likely attributable to the latter.[1,2]

In order to elucidate the above oxidation mechanism and lay down a basis for discriminating between proposed cation radicals, we have undertaken an electrochemical and EPR study of some suitable model compounds, chosen from among aminoanthraquinone dyes, in particular 1,4-diamino-9,10-anthraquinone (Disperse Violet 1) and 1,4-bis(methylamino)-9,10-anthraquinone (Disperse Blue 14). Our aim has been to obtain high-resolution spectra of cations and anions of these compounds, in order to map their spin-density patterns.



Compound	R^1	R^2	R^3	R^4
Disperse Violet 1 (DV1)	NH_2	R^1	H	H
Disperse Blue 14 (DB14)	$NH(CH_3)$	R^1	H	H
Ametantrone (AM)	$NH(CH_2)_2NH(CH_2)_2OH$	R^1	H	H
CL-55 343 (AMN-1)	$NH(CH_2)_2NH(CH_3)_2$	R^1	H	H
Mitoxantrone (MXH_2)	$NH(CH_2)_2NH(CH_2)_2OH$	R^1	OH	OH
Disperse Red 9 (DR9)	$NH(CH_3)$	H	H	H
Disperse Red 15 (DR15)	NH_2	OH	H	H
p-Phenylenediamine (PPD)	H	—	—	—
Tetramethyl-PPD (TMPD) (Wurster's Blue)	CH_3	—	—	—

We report here the electrochemical behaviour of these compounds as examined by cyclic voltammetry and EPR characteristics as obtained from their radicals.

The two disperse dyes and the aminoanthracenedione drugs can be considered as *p*-quinones or as aromatic diamines. The former systems are known to undergo two-electron electrochemical reductions to hydroquinones *via* a semiquinone

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radical stage, whereas the latter ones undergo two-electron oxidation to diimines. In aqueous solution the reactions for diamines are complicated, since they can exist in various states of protonation depending on pH.[5] We have attempted to determine the degree of protonation, *i.e.* to identify all labile amino protons by suitable deuterium-exchange experiments.

We were especially interested to see whether the pattern of coupling constants from the aminoanthraquinone cations showed any resemblance to those obtained from the cation of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (Wurster's Blue) or derived structures in view of their partial structural similarity.

Experimental

1,4-Diamino-9,10-anthraquinone (Disperse Violet 1, DV1), 1,4-bis(methylamino-9,10-anthraquinone (Disperse Blue 14, DB14), 1-amino-4-hydroxy-9,10-anthraquinone (Disperse Red 15, DR15) and 1-methylamino-9,10-anthraquinone (Disperse Red 9, DR9) were purchased from Aldrich and used as obtained. The supporting electrolyte tetrabutylammonium tetrafluoroborate ($TBABF_4$, 0.1 M) was prepared by standard methods. Acetonitrile was dried over Al_2O_3.

The cation radicals for EPR measurements were generated *in situ* by electrochemical oxidation at room temperature, in a flow cell placed close to the cavity of the spectrometer. A continuous solvent flow of 2 ml min^{-1} was maintained for all radicals by an HPLC pump. For a detailed description of the electrochemical equipment, see refs. 6 and 7.

EPR spectra were recorded on a Bruker ER200 spectrometer with a modulation frequency of 25 kHz. The microwave power was kept below ~1.3 mW. All spectra were interpreted by interactive simulations, using Lorentzian line shape and constant linewidth to all lines. However, when two or more spectra were superposed each one was characterised by its own linewidth and intensity, eventually determined by the computer in the optimization.

Results

Cation radicals of aminoanthraquinones

Few papers in the EPR literature are concerned with radical cations of anthraquinones, in contrast to the number dealing with the corresponding radical anions.[8] Most cations are made from anthraquinols under strongly acidic conditions, often by the use of a mixture of acids and photolysis. These procedures result in protonated quinone radicals.[9–11] In the case of aminoanthraquinones there arises the additional possibility of protonating the amino groups.

In order to obtain cations, and simultaneously to control the degree of protonation, electrolytic oxidation is carried out in solvents such as acetonitrile or dichloromethane.

Cyclic voltammetry. Cyclic voltammograms of DV1 and DB14 show two reversible single electron oxidation waves, in agreement with an expected overall two-electron oxidation of diaminoanthraquinone. The voltammogram of DB14 is shown in Fig. 1. The standard potentials, shown in Table 1, are readily obtained directly from the voltammograms. For 1-amino-4-hydroxy-9,10-anthraquinone (DR15) and 1-methylamino-9,10-anthraquinone (DR9) the second oxidation wave is irreversible for DR15 at a scan rate equal to 1 V s^{-1} and for DR9 at a rate equal to 5 V s^{-1}.

Electron paramagnetic spectroscopy. EPR spectra of the cation radicals for the spectroscopic study were generated by controlled current electrolysis. The potential was checked to be at the first oxidation wave. In order to optimise the experimental parameters for maximum signal intensity the cations were first detected by overmodulating the EPR signal. The modulation amplitude was then brought down to 15–25 mG. Attempts to observe EPR spectra from the cations of DR9 and DR15 were unsuccessful.

At low-modulation amplitudes, high-resolution spectra of DV1 and DB14 in dry acetonitrile were observed as shown in Figs. 2 and 3(*a*), respectively. Both spectra exhibit an unusually large spectral extension (~42 G in case of DV1, **Ia**, and ~76 G in case and DB14, **IIa**), implying that they cannot be derived from semiquinone radicals. Fig. 2 reveals a triplet of about 1 G from two protons, a splitting of 4.5 G from two nitrogens and one of 5.5 G from four protons. A simulation shows immediately that the 5.5 G splitting is derived from two slightly different sets of protons (Table 2, **Ia**). The linewidth amounts to 169 mG in this case. Adding an extra set of two equivalent protons in the simulation decreases the linewidth to 147 mG and a splitting of 57

Table 1 Standard potentials of some α-aminoanthraquinone cation radicals *vs.* SCE in acetonitrile at room temperature

Compound	E^1_{ox}/V^a	E^2_{ox}/V^a	E_{pa}/V^a	E_{pc}/V^a	E^1_{ox}/V^b
DV1	0.72	1.22	—	—	—
DB14	0.54	1.04	—	—	—
DR15	—	—	1.08	0.82	1.01
DR9	—	—	1.18	1.02	1.16

[a] The potentials are reported relative to the reference electrode SCE taken with the ferrocenium–ferrocene couple as an internal standard (E° = 0.40 V *vs.* SCE in acetonitrile). [b] Published values from ref. 12 *vs.* SCE.

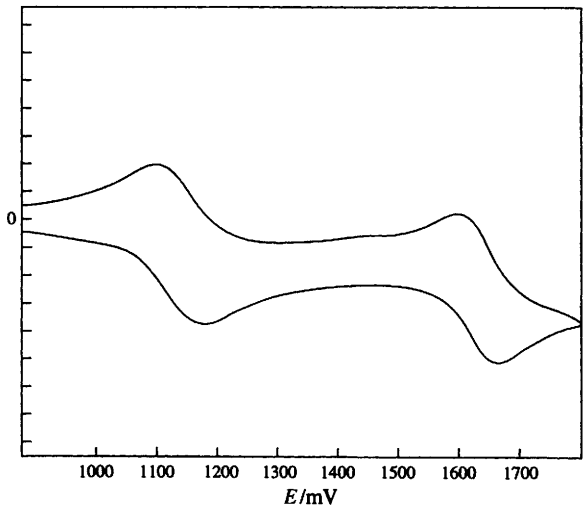


Fig. 1 Cyclic voltammogram of DB14 at a scan rate of 1 V s^{-1} in 0.1 M $TBABF_4$ and acetonitrile

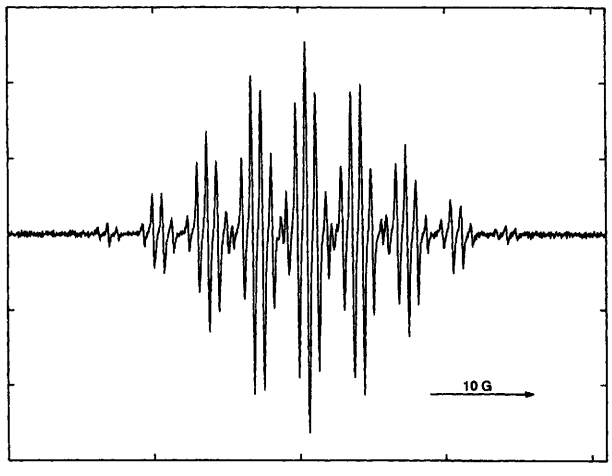


Fig. 2 The EPR spectrum of the cation radical of DV1 in acetonitrile

Table 2 Observed splitting constants (a_j/G) of aminoanthraquinone cations obtained from the radicals in dry acetonitrile. The constants are compared with similar constants obtained from Wurster's Blue-type cation radicals

		a_j/G							
Radical	Substituents	2	3	5	6	N	$2H^N$	$2H^N$	$6H^{Me}$
Ia	1,4-Diamino	0.957	0.957	*a*	0.057	4.495	5.577	5.443	—
IIa	1,4-Bis(methylamino)	1.048	1.048	*a*	0.096	5.294	6.010	—	6.759
PPD^{+} [b]		2.13	2.13	2.13	2.13	5.29	5.88	5.88	—
$TMPD^{+}$ [c]		1.97	1.97	1.97	1.97	6.99	—	—	6.76 [d]

[a] Not observed. [b] From ref. 13. [c] From ref. 14. [d] From 12 methyl protons.

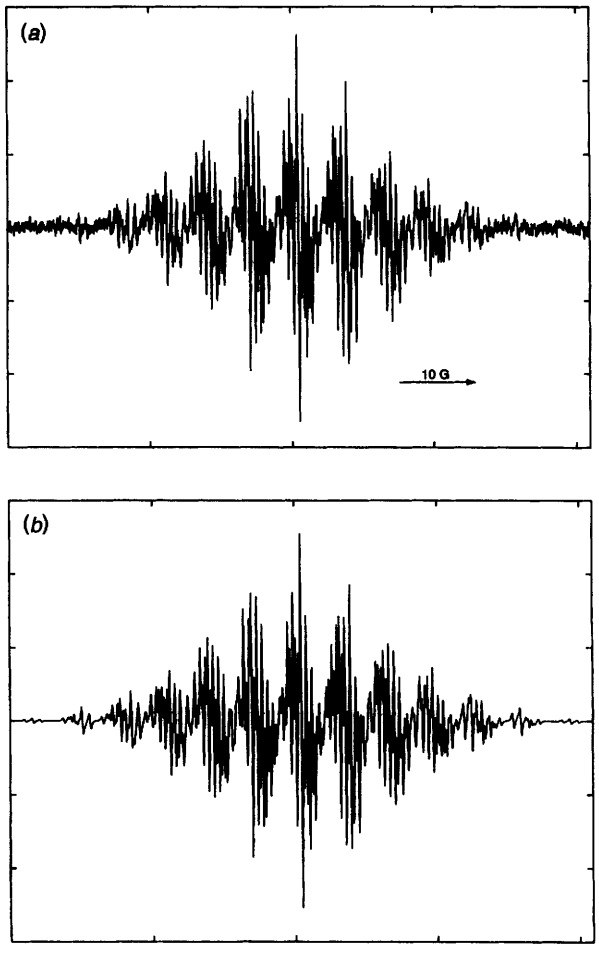


Fig. 3 The EPR spectrum of (*a*) the cation of DB14 in acetonitrile and its simulated counterpart (*b*). Note that the two outermost triplets in (*a*) disappear in the noise.

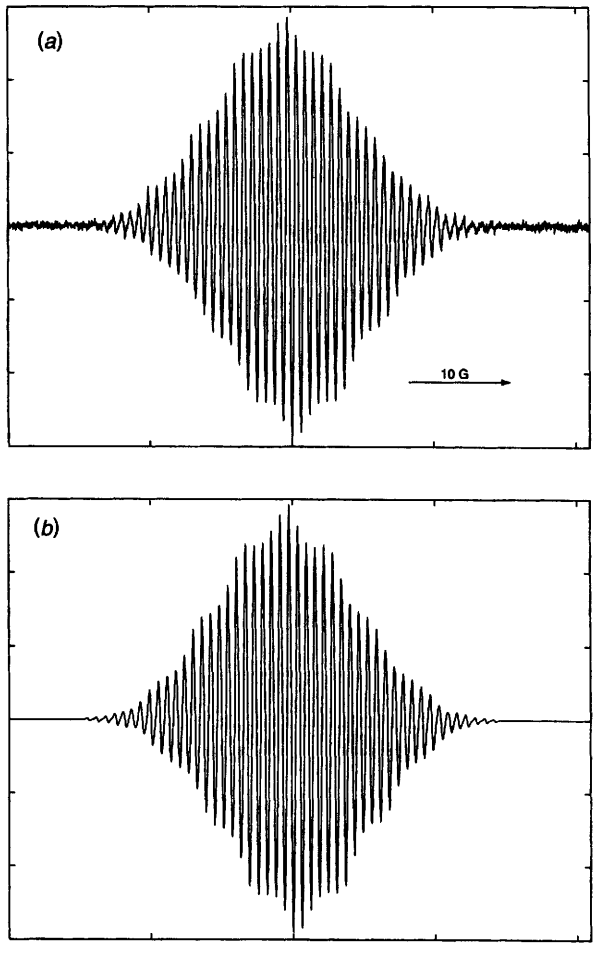


Fig. 4 The EPR spectrum of (*a*) the cation radical of DV1 in acetonitrile, with 25:25 μl D_2O–H_2O added to 100 ml solvent. The simulated counterpart (*b*), is made up of five radicals, the data of which are shown in Table 3 (**Ib**).

mG is obtained. The spectral fit improves by 14%. The spectrum thus comprises of five sets of equivalent nuclei: 2 H, 2 H, 2 H, 2 H and 2 N.

When the experiment is repeated with DB14, a complex spectrum appears from the corresponding cation (**IIa**) as shown in Fig. 3(*a*). DB14 has methylamino groups at C-1 and C-4, which more than doubles the number of lines as compared with the spectrum of Fig. 2. With the result of DV1 as a guide, the spectrum can be analysed in terms of four sets of nuclei: 2 H, 2 Me, 2 H and 2 N (Table 2), and the simulation shown in Fig. 3(*b*) is a close match of that in Fig. 3(*a*). As with the spectrum of radical **Ia**, adding a small splitting of 96 mG (triplet) decreases the linewidth from 247 mG to 190 mG and improves the fit by 21.6%.

In order to assign the observed splitting constants, exchange of labile amino protons was attempted by way of deuterium experiments. From preliminary experiments with dried solvents, it turned out that adding small amounts of H_2O to the acetonitrile solvent in fact gave slightly better-resolved spectra. Accordingly, we added 25 μl of D_2O and 25 μl of H_2O to 100 ml acetonitrile solution of DV1 and obtained the spectrum shown in Fig. 4(*a*). Careful analysis and detailed simulations [Fig. 4(*b*)] revealed that the spectrum is made up of spectra from five radicals differing in that they contain different numbers of deuterium atoms (0–4). A careful simulation furnishes the constants shown in Table 3 (**Ib**). It is obvious from the table that the splitting constants of about 5.5 G all belong to the amino protons. Repeating the experiment with 50 μl of D_2O added to dried acetonitrile gave rise to the spectrum **Ic** comprised of three radicals with, respectively, 4, 3 and 2 amino deuterons (Table 3). Some water, still left in the dried solvent and remaining in the D_2O sample, and the amino protons from the parent substance, make the exchange incomplete. Conducting the same experiment with the cation of DB14 and with 50 μl of D_2O added gave rise to a spectrum (**IIb**) comprised of two radicals with, respectively, 2 and 1 of the available amino protons exchanged (Table 3). All amino protons are hereby singled out

Table 3 Observed splitting constants (a_j/G) of deuteriated and non-deuteriated cations obtained from the radicals in acetonitrile with some D_2O–H_2O added. The stated amounts were added to 100 ml samples of acetonitrile, which was dried in cases where only D_2O was added. The deuteriation is solely confined to labile amino protons

			a_j/G										
Radical	D_2O:H_2O/μl		2	5	6	N	$2D^N$	$2D^N$	D^N	$2H^N$	$2H^N$	H^N	$6H^{Me}$
Ib	25:25	4D	1.121	*a*	0.136	4.383	0.930	0.849	—	—	—	—	—
		3D	1.050	*a*	0.110	4.431	0.886	—	0.856	—	—	5.535	—
		2D	1.023	*a*	0.097	4.479	0.841	—	—	5.462	—	—	—
		1D	0.965	*a*	0.094	4.475	—	—	0.821	5.473	—	5.568	—
		0D	0.924	*a*	0.099	4.457	—	—	—	5.567	5.539	—	—
Ic	50:0	4D	0.954	*a*	0.053	4.376	0.847	0.880	—	—	—	—	—
		3D	0.968	*a*	0.078	4.409	0.840	—	0.890	—	—	5.536	—
		2D	0.939	*a*	0.061	4.464	0.838	—	—	5.491	—	—	—
IIb	50:0	2D	1.060	*a*	0.086	5.246	0.945	—	—	—	—	—	6.774
		1D	1.037	*a*	0.065	5.291	—	—	0.928	—	—	5.980	6.781

[a] Not observed.

Table 4 Relative amount (rel. amount) of the various radical cations obtained after deuterium exchange of amino protons. The figures stated derive from calculations based on the D_2O:H_2O ratio (v/v). Also stated are the corresponding relative intensities (rel. int.) and the intensity (int.) of the most intense line in a radical as obtained from the simulation program. Spin comb. comprises the number of spin components making up each individual spectrum

Radical	Linewidth	D	H	Rel. amount	Rel. int.	Int.	Spin comb.
				50:50[b]			
Ib	0.146	4	—	6.3	7.7	15.4	11 664
	0.205	3	1	25.0	25.0	43.3	7 776
	0.171	2	2	37.5	34.9	100.0	5 184
	0.162	1	3	25.0	24.7	71.7	3 456
	0.154	—	4	6.3	7.7	41.4	2 304
				85:15[b]			
Ic	0.132	4	—	52.2	55.3	100.0	11 664
	0.123	3	1	36.8	34.5	31.5	7 776
	0.131	2	2	9.8	10.2	11.6	5 184
				85:15[b]			
IIb	0.195	2	—	72.3	76.4	100.0	82 944
	0.238	1	1	25.5	23.6	18.4	55 296
	a	—	2	2.2	—	—	36 864

[a] Radical not detectable by simulation. [b] D_2O:H_2O ratio (v/v).

and only the smaller proton splittings of Table 2 are unassigned. We tentatively assign the splitting of 1 G to the protons at C-2 and C-3, the splitting of 57 mG (96 mG) to the protons at C-6 and C-7, with no splitting observed from the protons at C-5 and C-8.

In order to check the relative amounts observed of the various radical isomers, estimates of concentration are shown in Table 4. The relative intensities are obtained in the simulation procedure by multiplying the number of spin combinations for a radical by the linewidth squared and a scaling factor involving the intensity of the most intense line of each radical. These figures should now be compared with those calculated from the D_2O:H_2O ratio (Table 4). The correspondence is excellent, demonstrating that highly complex simulations can be performed with confidence, *cf.* Fig. 4(*a*) and (*b*), and Table 3, **Ib**.

The results shown in Table 3 exhibit a marked H/D isotope effect. Thus, for radical **Ib** the nitrogen splitting, a_N, and the splittings from remaining amino protons decrease in magnitude when more and more protons are exchanged with deuterons. The opposite effect is observed for a_2 and a_6. A deuteron splitting increases in magnitude with the number of deuterons present.

Finally, if we compare the data of Table 3 for the cations **I** and **II** with those obtained from *p*-phenylenediamine (PPD)[13] and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD),[14] an obvious similarity appears (Table 2). Both the aminoanthraquinone and the *p*-phenylenediamine cations exhibit large nitrogen and amino proton splittings of 4.5–7 G. When the ring system becomes extended, as in the case of the aminoanthraquinones, we observe decreased values for ring proton splittings. We can conclude, that the cations observed by the loss of one electron from 1,4-diaminoanthraquinones can be regarded as aromatic diamino radicals and the quinone moiety as a mere extension of the radical skeleton (*cf.* Scheme 1 below).

Anion radicals of aminoanthraquinones

Only few EPR studies have dealt with the radicals of aminoanthraquinones. Ciureanu *et al.*[15] used electrolytic reduction in studying the anion radical of 2-aminoanthraquinone, whereas Pedersen[16] generated the same radical chemically. Bock *et al.*[17] studied the contact ion-pair between alkali metal cations and the anion radical of 1-aminoanthraquinone. Nguyen and Gutierrez[4] investigated the anion radicals of mitoxantrone and of an analogue of ametantrone by EPR spectroscopy and cyclic voltammetry.

We have generated the free anion radicals of DV1 and DB14 by electrochemical reduction in DMF, as well as the one of DV1 in DMSO. The high-resolution spectra observed had linewidths in the range 34–71 mG. The spectra of DV1 have been published previously.[18] By interactive simulation complete agreements were obtained between the experimental and the theoretical spectra. The obtained splitting constants are shown in Table 5, together with the results for the anion radical of anthrasemiquinone (Asq) for comparison. Among the constants observed, only those from nitrogen and from the methyl protons, in the case of DB14, are assigned unequivocally. The remaining constants are assigned as follows. We assume the spin densities at C-5 to C-8, by and large, to be undisturbed by

Table 5 Observed splitting constants (a_j/G) of some aminoanthraquinone anion radicals. The radicals were obtained by one-electron reduction in dried DMF or DMSO

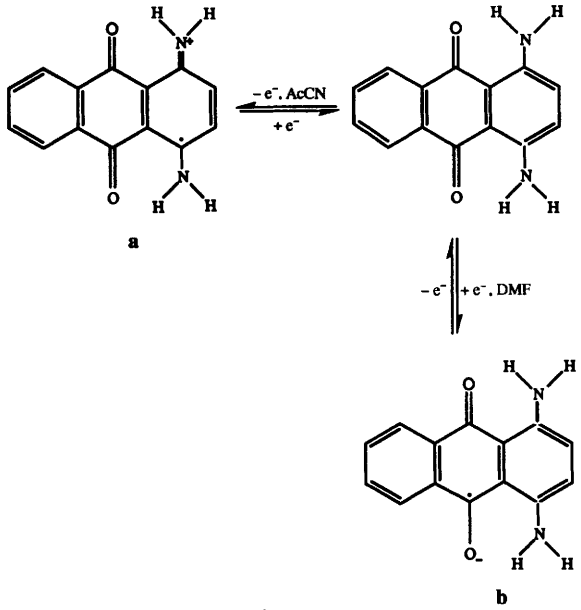
Anion radical	a_j/G								Lw/mG	Solvent
	1,4	2,3	5,8	6,7	N	$2H^N$	$2H^N$	$6H^{Me}$		
Asq	0.279	0.981	0.279	0.981	—	—	—	—	41	DMF
	0.301	0.978	0.301	0.978	—	—	—	—	34	DMSO
1,4-Diamino-Asq (DV1)	—	1.913	0.241	0.953	0.701	0.481	0.016	—	39	DMF
	—	1.917	0.278	0.945	0.762	0.534	0.029	—	52	DMSO
1,4-Bis-(methylamino)-Asq (DB14)	—	2.633	0.200	0.842	0.625	—	0.046	0.439	71	DMF
CL-55,343 (AMN-1)[b]	—	2.29	0.50	2.29	0.25	—	—	—	—	DMSO
Mitoxantrone[b]	—	4.15	0.289[c]	1.924	0.80	—	—	—	—	DMSO

[a] Asq = anthrasemiquinone. [b] From ref. 4. [c] Hydroxy proton splitting constant.

the presence of the amino groups at C-1 and C-4, *i.e.* the proton splitting constants are taken to be of similar magnitude to those observed from the anthrasemiquinone anion radical (Table 5). The protons at C-2 and C-3 are expected to show the largest splitting constants. Remaining constants are then assigned to the amino protons.

Discussion

Clearly, the cation radicals of the 1,4-diaminoanthraquinone dyes DV1 and DB14 must be considered as aromatic diamino radicals, sometimes termed semiquinonediimines, *i.e.* they appear after electrochemical one-electron oxidation according to Scheme 1, **a**. Large splittings (4.5–7 G) observed from the



a **b**

Scheme 1

nitrogen atoms and the amino protons, and small (~1 G) or missing splittings from the aromatic protons, prove the cations to be of similar type to the Wurster's Blue cation.

This result excludes unequivocally the possibility that the single-line EPR spectrum observed from mitoxantrone is derived from a cation obtained from a single-electron oxidation, *i.e.* being derived from the $MXH_2^{\bullet+}$ radical [eqn. (1)]. We expect this radical to have constants close to those of radical **II** from DB14.

The reasoning is as follows, where we consider ametantrone at first. It differs from DB14 only by having 2-(hydroxyethyl)aminoethyl substituents at the amino groups, instead of methyl groups. The perturbations at the amino sites, ethyl *versus* methyl, are therefore comparable, giving ametantrone and DB14 closely related EPR spectra. Mitoxantrone differs from ametantrone by having hydroxy groups at C-5 and C-8. However, we know from the data of Table 2 that these groups are placed at positions with non-detectable spin populations. Accordingly, the cation spectrum from a one-electron oxidation of mitoxantrone should exhibit close resemblance to the one of ametantrone.

Large spin densities, confined to the aromatic amino groups of mitoxantrone, suggest that the most probable sites of an enzymic attack would be at these groups. Furthermore, the observation that the absence of phenolic hydroxy groups (as in ametantrone) does not prevent the oxidation reaction is in complete accordance with the subordinate role attributed above to the hydroxy groups, which are placed at positions with spin densities close to zero.

The amino protons, all unambiguously identified by deuterium exchange, appear to be placed in positions of near magnetic equivalence (*cf.* $a_{NH} = 5.577 \approx 5.443$, Table 2) for DV1. Substituting an amino proton with a methyl group (DB14) does not affect this observation. In fact, the constants of methyl protons are seen to be nearly identical to the ones observed from corresponding NH protons they replace, *cf.* **Ia** ⟶ **IIa** and PPD^+ ⟶ $TMPD^+$ (Table 2).

Finally, the single-line spectrum mentioned above, is most probably derived from the cation of the MH_2 metabolite, known to be generated in the enzymatic HRP-oxidation of mitoxantrone.[1]

In contrast to the cation radicals of DV1 and DB14 their corresponding anion counterparts clearly belong to the well characterised class of semiquinone radicals, obtained by the electrochemical one-electron reduction according to Scheme 1, **b**, *i.e.* the unpaired electron is delocalised over the entire quinone skeleton, with the appearance of many splitting constants, mostly of magnitude less than ~3 G.

Furthermore, in contradistinction to the result found for the cation radicals the amino protons in a particular amino group of anion radicals are found to be magnetically non-equivalent, as is obvious from the result of DV1, where no two proton splittings are of similar magnitude (Table 5).

Nguyen and Gutierrez[4] used electrochemical reduction with DMSO as the solvent to generate the anion radicals of mitoxantrone and of the Lederle compound CL-55,343 termed AMN-1, an analogue of ametantrone. They obtained the data shown in Table 5. The EPR spectrum of AMN-1 showed hyperfine lines superimposed on a large singlet. Since they do not report splittings from any of the amino protons, which is in contrast to our findings, their simulation may need re-evaluation. Furthermore, they assign large splittings (2.29 G) to the protons at C-6 and C-7, as well as to those at C-2 and C-3. We observe only two proton splittings of a magnitude ~2 G,

Their result for mitoxantrone can similarly be questioned, because (*a*) they assign splittings to the hydroxy protons but not to any protons of the amino groups, and (*b*) the splittings assigned to the protons at C-2 and C-3 seem unrealistically large (4.15 G).

Summarising, one-electron oxidation of 1,4-diaminoanthraquinones leads to diamino cation radicals, and the

corresponding reduction leads to anthrasemiquinone anion radicals.

Amino protons of cations are found magnetically near-equivalent, while a marked non-equivalence appears in the case of anions. Thus, we observe H-bond formation between a proton in each amino group and the carbonyl oxygens in the latter case, reinforced by the negatively charged carbonyl groups.

The linewidths are approximately three times larger for cations than for corresponding anions. Unresolved splittings from C-5 and C-8 for the cations may be part of an explanation. Also the use of different solvents may lead to different degrees of line broadening. The temperature dependence of the spectra has not been investigated, due to the difficulty of carrying out temperature studies on radicals generated under flowing conditions.

The combination of electrochemistry and EPR spectroscopy with the generation of radical anions and cations *in situ* has demonstrated its advantages, in that (*a*) acid conditions can be avoided, which for cations is essential for controlling the degree of protonation, (*b*) the addition of small amounts of D_2O–H_2O to the acetonitrile solvent flow has made possible the identification of labile amino protons, and (*c*) the identity of a given radical is ensured by the electrochemical one-electron transfer and by observation of high resolution EPR spectra, containing highly valuable information. This information, however, can be used with confidence only when obtained by way of advanced computer simulations.

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