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DIMINISHED SUPEROXIDE ANION GENERATION BY REDUCED 5-IMINODAUNORUBICIN RELATIVE TO DAUNORUBICIN AND THE RELATIONSHIP TO CARDIOTOXICITY OF THE ANTHRACYCLINE ANTITUMOR AGENTS *

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Abstract—5-Iminodaunorubicin, when reductively activated, produces single strand scission in PM2-CCC-DNA by a mechanism which proceeds via production of superoxide anion and hydroxyl radicals. Under comparable conditions, 5-iminodaunorubicin produces much less nicking than daunorubicin. With the aid of *N*-cyclohexyl-5-iminoquinizarin, as a model, assignment of polarographic waves to the quinone moiety of 5-iminodaunorubicin was possible. The electrochemical results indicate that 5-iminodaunorubicin is more difficult to reduce than daunorubicin and that reoxidation of the reduced form, 5,11-dihydro-5-iminodaunorubicin, is much more difficult than reoxidation of reduced daunorubicin. The latter conclusion is supported by independent chemical studies. By comparison of 5-iminodaunorubicin with daunorubicin and *N*-cyclohexyl-5-iminoquinizarine, the unusual stability of the reduced 5-iminodaunorubicin, is tentatively attributed to strong hydrogen bonding. The results suggest a correlation between the diminished generation of superoxide anion by 5-iminodaunorubicin and its observed suppressed cardiotoxicity relative to other anthracyclines.

The antibiotics, daunorubicin (**1a**), and adriamycin (**1b**), (Fig. 1), exhibit clinically useful activity against a range of neoplasms including leukemia and several solid tumors [1]. Biochemical evidence indicates that their antineoplastic properties are due largely to strong intercalative binding to nucleic acids with consequent inhibition of DNA replication and/or RNA synthesis [1–3]. Currently, their clinical effectiveness is severely limited by dose-related cardiotoxicity [4,5]. Thus, there is an urgent need to understand the molecular basis of the cardiotoxicity and to modify the anthracyclines structurally to obviate this side effect. Bachur *et al.* [6] have obtained evidence that the anthracyclines undergo a cyclic redox reaction *in vivo* leading to production of superoxide anion. There is accumulating evidence that relates the cardiotoxicity of the anthracyclines to the generation of such free radicals, especially $O_2^{\cdot-}$ and $OH^{\cdot}$ in cardiac tissue [7–10], which is deficient in the protective enzymes, superoxide dismutase and catalase [11]. We report studies on the reactions of the chromophore-modified anthracycline, 5-iminodaunorubicin, with DNA and in particular how its diminished production of superoxide anion and consequent reduced single strand cleavage of CCC-DNA (covalently closed circular) relates to its observed lower cardiotoxicity compared with the parent antibiotic.

MATERIALS AND METHODS

Daunorubicin was purchased from CalBiochem, La Jolla, CA. 5-Iminodanuorubicin was prepared at SRI, Menlo Park, CA 94025, under contract to the National Cancer Institute (Contract No1-CM-33742). Superoxide dismutase (EC 1.15.1.1) was from the Sigma Chemical Co., St. Louis, MO 63178, and catalase (EC 1.11.16) (beef liver) was from the Aldrich Chemical Co., Milwaukee, WI 53233. PM2-CCC-DNA was prepared as described previously [12]. Other materials used were reagent grade, except for acetonitrile (spectrophotometric grade).

Stability of reduced 5-iminodaunorubicin toward oxygen or H_2O_2. The progress of reduction of 5-iminodaunorubicin (λ_{max} 545 nm, ε 12,714) by 100 μl of 0.1 M $NaBH_4$ was followed spectrophotometrically. The initial purple color changed to the yellow (λ_{max} 434 nm, ε 8567) of the reduced form. The latter solution, when exposed to atmospheric oxygen for 16 hr or to a few drops of 30% hydrogen peroxide, showed no spectral change.

Preparation of N-*cyclohexyl-5-iminoquinizarin.* A mixture of quinizarin (0.72 g, 3 mM), toluene (10 ml), and a catalytic amount of *p*-toluenesulfonic acid was placed in a three-necked round bottom flask fitted with a condenser and an addition funnel. After the mixture was brought to reflux, freshly distilled cyclohexylamine (0.3 g, 3 mM) in 2 ml toluene was added dropwise to the refluxed reaction mixture. After the addition, the reaction was kept refluxing for 10 hr before it was cooled to room temperature. The reaction product was dissolved in benzene and was subjected to silica gel column chromatography with benzene eluent. The deep purple fraction was collected, concentrated and subjected to this chromatography again. Deep purple

* Studies related to Antitumor Antibiotics. Part XVIII.

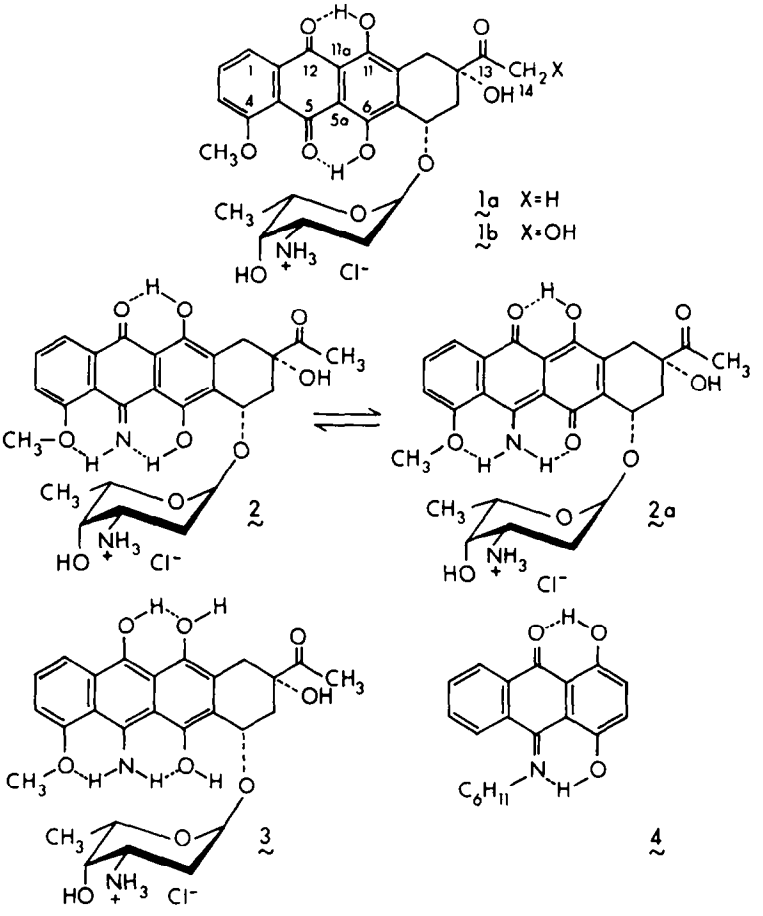

Fig. 1. Structural formulae of daunorubicin (**1a**) adriamycin (**1b**) 5-iminodaunorubicin (**2**) 5, 11-dihydro-5-iminodaunorubicin (**3**) and *N*-cyclohexyl-5-iminoquinizarin (**4**).

needles were isolated with 0.48 g (51 per cent yield), m.p. 160–161°; n.m.r. ($CDCl_3$) δ 1.15–2.20 (M, 10 H of cyclohexyl), 3.50 (M, 1 H, methine hydrogen), 7.08 (S, 2 H aromatic), 8.20 (M, 2. H aromatic), 11.40 (d, 1 H OH) and 15.65 (S, 1 H OH); i.r. (V Nujol) 1610 (C=O), 1590 cm^{-1} (C=N), MS, *m/e* (relative intensity) 321 (M^+, 100), 278, ($M^+ - C_3H_7$, 85). Anal. calc. for $C_{20}H_{19}NO_3$ (mol. wt. 321) = C, 74.76; H, 5.92; N, 4.36; O 14.95. Found: c, 74.53; H, 5.86; N, 4.25; O, 15.05.

Ethidium fluorescence assay for superoxide anion generation by nicking of DNA by reductively activated anthracyclines. All fluorescence measurements were performed on a G. K. Turner & Associates model 430 spectroflourometer equipped with a cooling fan to reduce fluctuations in the xenon lamp. Wavelength calibration was performed as described in the manual for the instrument. One centimeter square cuvettes were used. The excitation wavelength was 525 nm and the emission wavelength was 600 nm. Medium sensitivity (× 100 scale) was generally used and water was circulated between the cell compartment and a thermally regulated bath at 22°. The reactions were performed at 37° in a total volume of 200 µl containing 50 µl of potassium phosphate buffer (pH 7.0), 1.02 A_{260} units of PM2-CCC-DNA (92% CCC), 9×10^{-5} anthracycline, and 5.3×10^{-3} M sodium borohydride. The concentrations of other components used are given in the legends to the figures. At intervals, 20-µl aliquots from the reaction mixture were added to 2.3 ml of the ethidium assay mixture, which was 20 mM potassium phosphate (pH 11.8), 0.4 mM EDTA and 0.5 µg/ml of ethidium bromide. The fluorescence of the diluted solution was measured. The solution was then heat denatured at 96° for 4 min, cooled in ice, and equilibrated in a water bath at 22° for 5 min. The fluorescence of the solution was again measured. The conversion of PM2-CCC-DNA to nicked circular DNA results in a 30 percent increase in fluorescence in the pH 11.8 assay solution. After heat denaturation and cooling, the CCC-DNA returns to register while the formation of nicked DNA is revealed by a complete loss of fluorescence. As additional controls, 20 µl of the reaction mixtures for the two anthracyclines were added to 2.3 ml of a solution of the assay medium without ethidium, which showed no fluorescence at 525 nm.

Electrochemical determination of redox behavior. Polarographic analysis and cyclic voltammetry were carried out as described previously [13,14]. The drop time was controlled at 2 sec. All potentials were measured and are reported with respect to the aqueous saturated calomel electrode. Aqueous solutions of 5-iminodaunorubicin were prepared in 0.1 M potassium phosphate buffer (pH 7.0) and 0.1 M KCl supporting electrolyte. Other solutions were CH_3CN–H_2O (90 : 10, v/v) buffered with 0.01 M phosphate buffer

and supporting electrolyte as above. All solutions were thermostated at 37.5°. Triton X-100 (Rohm & Haas, Philadelphia, PA) in 1% aqueous solution was used as a maximum suppressor. All potentials were measured and are reported with respect to the aqueous saturated calomel electrode. Potentials in the CH_3CN–H_2O (90 : 10) solvent, therefore, include a junction potential between solvents and should not be compared directly with potentials in aqueous solutions.

The *N*-cyclohexyl-5-iminoquinizarin was not sufficiently soluble for study in aqueous solutions and, therefore, was studied only in the CH_3CN–H_2O (90 : 10) medium. All other conditions for these solutions were identical to those used for 5-iminodaunorubicin.

RESULTS

Daunorubicin, **1a** (9×10^{-5} M), when reduced *in situ* with $NaBH_4$ (5.3×10^{-3} M), produces 90 percent nicking of PM2-CCC-DNA in 20 min at 37° (pH 7.0) [15]. Under comparable conditions (same concentration of anthracycline, $NaBH_4$, 37°, pH 7.0), the 5-iminodaunorubicin (**2**) produces much less nicking, i.e. 25 percent in 20 min. Reduction of the anthracyclines prior to their addition to the DNA gave slightly more rapid states of cleavage but approximately in the same ratio, i.e. 93 percent nicking in 20 min for daunorubicin and 32 percent nicking for 5-iminodaunorubicin (Fig. 2).

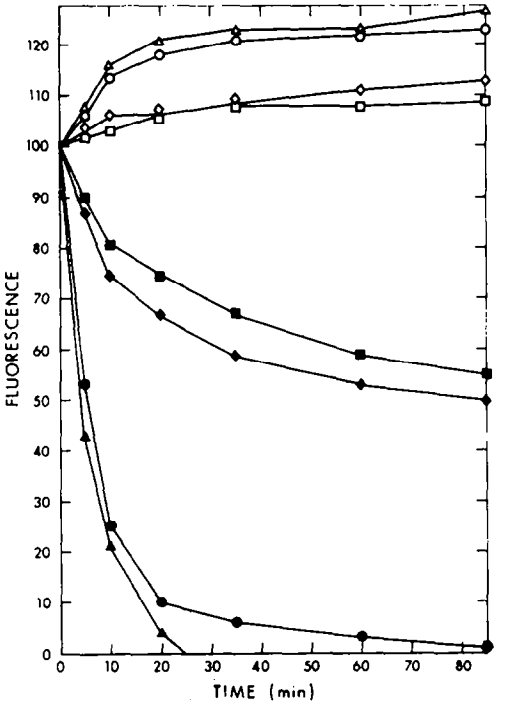

Fig. 2. Comparison of the effects of the reduced forms of 5-iminodaunorubicin and daunorubicin in the single strand scission of PM2-CCC-DNA. Reactions were performed at 37° in 0.05 M potassium phosphate buffer (pH 7.0) and contained 1.0 A_{260} unit/ml of PM2-CCC-DNA (92% CCC). The before heat fluorescence readings are shown as open symbols and the closed symbols are fluorescence readings after the denaturation at 96° for 4 min and rapid cooling. Additional components were: (□—□) 9×10^{-5} M 5-iminodaunorubicin and 5.3×10^{-3} M $NaBH_4$; (◇—◇) 9×10^{-5} M pre-reduced 5-iminodaunorubicin; (○—○) 9×10^{-5} M daunorubicin and 5.3×10^{-3} M $NaBH_4$; and (△ — △) 9×10^{-5} M pre-reduced daunorubicin.

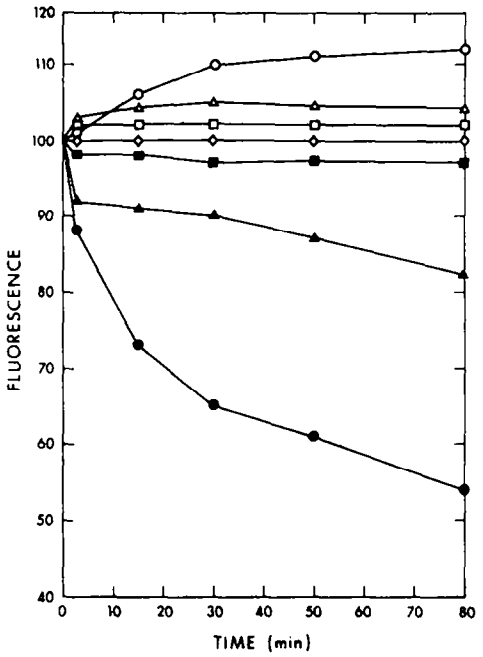

Fig. 3. Single strand scission of PM2-CCC-DNA by reduced 5-iminodaunorubicinand its selective inhibition by enzymes and radical scavengers. Reactions were carried out as described in the legend to Fig. 2 and contained 9×10^{-5} M 5-iminodaunorubicin and 5×10^{-3} M $NaBH_4$. Additional components were:(◇—◇) 0.6 M isopropyl alcohol or 0.225 M sodium benzoate; (□—□) 5×10^{-6} g/ml of catalase; (△—△) 5×10^{-5} g/ml of superoxide dismutase; and (○—○) none.

The nicking of DNA by the reduced form of **2**, presumed to be **3** or the tautomeric leuco form, is markedly suppressed by the addition of superoxide dismutase or by catalase and more efficiently by isopropyl alcohol or sodium benzoate (Fig. 3).

Polarography of aqueous solutions of **2** in the absence of maximum suppressor gave three well-defined but unusually shaped waves which appeared in differential pulse polarography as sharp peaks ($E_{\frac{1}{2}} = E_p = -0.67$ V, -0.94 V, -1.17 V). With addition of two drops of Triton solution to 10 ml of test solution these anomalous effects disappeared; further addition of Triton had no effect. Polarography then yielded a single wave ($E_{\frac{1}{2}} = -0.657 \pm 0.005$ V) of a slightly distorted form very similar to that of daunorubicin [12,13] (Fig. 4A). Pulse and differential pulse polarography showed that this reduction, like that of daunorubicin, was due to two cathodic processes separated by less than 50 mV rather than a single cathodic process. Cyclic voltammetry, which was run without maximum suppressor (Fig. 5), showed the same cathodic peaks but no significant anodic peaks. Restriction of the potential range to that of the processes I, -0.60 V to -0.85 V, confirmed the absence of an anodic counterpart of I, a significant difference between the behavior of **2** and that of daunorubicin.

Polarography of **2** in CH_3CN–H_2O (90:10) (Fig. 4B) showed three waves, which appeared as peaks in

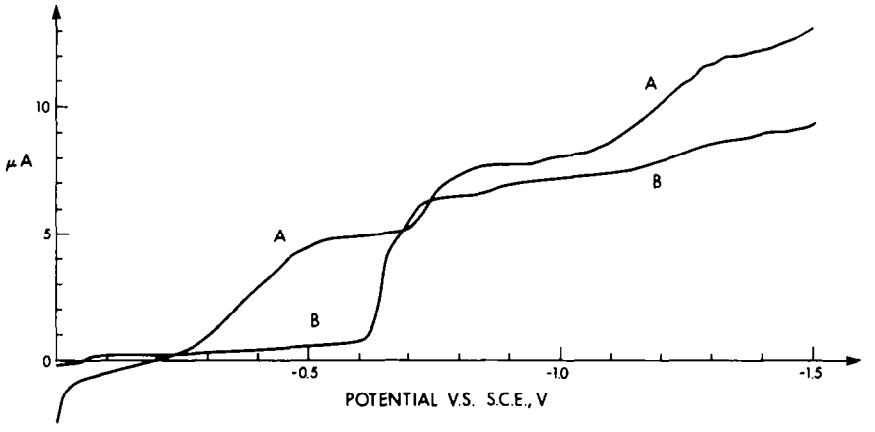

Fig. 4. Polarography of iminodaunorubicin. Curve A, aqueous; curve B, CH_3CN–H_2O (90 : 10). Aqueous phosphate buffer, pH 7.1, 37.5°, concentration 0.13 m-mol/l.

differential pulse polarography: $E_t = E_p = -0.38$ V, -0.75 V and -1.27 V. Cyclic voltammetry (Fig. 6) shows that only the process at -0.75 V has an anodic counterpart even in this partially aqueous medium.

Polarography of the model compound *N*-cyclohexyl-5-iminoquinizarin (4) in CH_3CN–H_2O (90 : 10) gave two waves ($E_t = -0.52$ V, -1.52 V) which appear as broad peaks in differential pulse polarography ($E_p = -0.58$ V, -1.25 V) and one major double wave ($E_t = -0.72$ V, -0.84 V). Cyclic voltammetry gave well-defined waves of two cathodic processes ($E_p = -0.76$ V, -0.86 V, at 500 mV/sec and range 0 to -1.5 V) and one anodic process ($E_p = -0.74$ V). Use of a restricted potential scan range and slower scan rates permitted resolution of the anodic process into two components, the anodic counterparts of the two cathodic processes.

DISCUSSION

Data from several *in vitro* tests indicate an alteration in the DNA interactive properties of **2** compared with **1a** that may be significant in its molecular mechanism of action [16]. 5-Iminodaunorubicin shows virtually complete loss of mutagenicity to *Salmonella typhimurium* in the Ames test and a lower elevation of T_m of helical DNA (ΔT_m 6.25° compared with 11.2° for **1**) [16].

5-Iminodaunorubicin (**2**), when reductively activated, nicks PM2-CCC-DNA at a substantially diminished rate compared with the parent anthracycline **1a**. The results of the selective inhibition of the nicking process confirm the intermediacy of superoxide anion, hydrogen peroxide and hydroxyl radicals. Since the cleavage reaction proceeds by a mechanism closely similar to that which is operative for daunorubicin, the

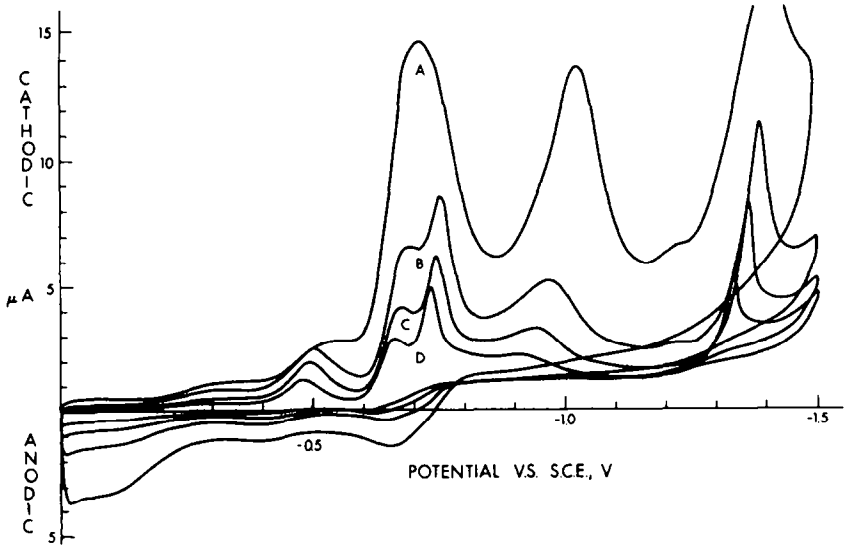

Fig. 5. Cyclic voltammetry of aqueous iminodaunorubicin. Curve A, 500 mV/sec; B, 200 mV/sec; C, 100 mV/sec; and D, 50 mV/sec. Aqueous phosphate buffer, pH 7.1, 37.5°, concentration 0.18 m-mol/l.

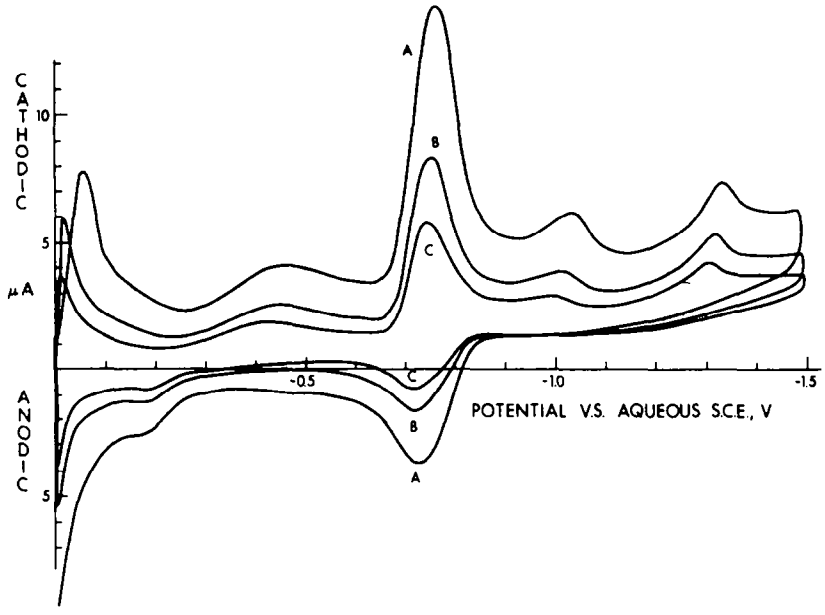

Fig. 6. cyclic voltammetry of iminodauorubicin in CH_3CN–H_2O (90 : 10). Curve A, 500 mV/sec; B, 200 mV/sec; and C. 100 mV/sec. Shape of processes I does not change down to 20 mV/sec. Phosphate buffer. 37.5°.

observed differences in rate of superoxide anion generation could be due to (i) a slower rate of reduction of **2** compared with **1a** and/or (ii) a slower rate of reoxidation of the reduced form of **2** relative to that of **1a**.

In aqueous solution, the reduction of **2** parallels that of daunorubicin (**1a**) [12]. Reduction of **1a** is significantly easier; the comparable half-wave potentials are −0.58 V for daunorubicin and −0.66 V for **2**. The reoxidation observed with **1a** [14], however, is not observed with **2**. Chemical demonstration of this was possible; aqueous solutions of **3**, the reduced form of **2**, were quite stable to exposure to atmospheric oxygen for 16 hr. Even treatment of **3** with dilute hydrogen peroxide resulted in only slight reoxidation. In contrast, the reduced form of danuorubicin is air sensitive and solutions of it are completely reoxidized extremely rapidly [14]. Therefore, we may conclude that reduction of **2** in aqueous solution is more difficult than for daunorubicin and that the reoxidation of the reduced form is very much more difficult.

The model compound **4** is similar in structure to the chromophore of **2** and shows, under the same conditions of cyclic voltammetry, a first cathodic peak identical to that of **2**. This process, therefore, cannot be related to the sugar moiety or the side chain carboxyl of 5-iminodaunorubicin and supports the assignment of the electrochemically active group of the iminiquinone moiety in **2** and **4**. The reduced form of **4**, unlike **3**, is readily reoxidized.

It appears likely that factors tending to stabilize the reduced 5-iminodaunorubicin (**3**) and absent in the reduced form of **4** include the additional hydrogen bonding of the NH_2 group to the neighboring oxygen functions.

Nuclear magnetic resonance (n.m.r.) and carbon magnetic resonance spectroscopy (c.m.r) have provided evidence for three hydrogren bonds contributing to the stabilization of **2** [16]. These spectral data indicate a stronger C_{12}—O—C_{11} hydrogen bond in **2** relative to **1a** and may contribute to the greater stability of **2** and consequent difficulty of reduction compared with **1a**. There was no evidence of hydrolysis of **2** to **1a** [Ref. 16 and present work] under the conditions employed so that the diminished rate of reduction of **2** is not due to prior slow hydrolysis to **1a**.

Hydrogen bonding between the imine nitrogen and the adjacent hydroxyl group undoubtedly contributes to stabilization in this series; while the model compound **4** could be readily prepared, the corresponding imino 9,10-anthraquinone could not be isolated. An additional factor tending to alter the redox potential of **2** (making it more difficult to reduce than **1a**) is the contribution of the tautomer **2a** for which n.m.r. and c.m.r. evidence has been obtained [16]. While these factors make **2** more difficult to reduce than **1a**, they also make the reduced 5-iminodaunorubicin once formed more difficult to reoxidize than the corresponding reduced form of the parent antibiotic. Apart from the additional hydrogen bond of the NH_2 group in **3**, the strengthening effect of the greater electron-releasing ability of the 5-N function on the C-12 O—H bond [16] will also contribute.

In conclusion, if 5-iminodaunorubicin is less subject to the cyclic reduction-reoxidation *in vivo* that has been demonstrated for the anthracyclines and other quinone-containing antibiotics, it will be much less efficient in the generation of superoxide anion. This may suggest a correlation between the diminished generation of superoxide anion by **2** with the observed suppressed cardiotoxicity of **2** [10,16] relative to daunorubicin and adriamycin in view of the known deficiency of superoxide dismutase and catalase in cardiac tissue [11].

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