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Metal Ion Binding to Daunorubicin and Quinizarin

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The anthracycline containing antibiotic daunorubicin binds metal ions with stabilities similar to those of quinizarin (1,4-dihydroxyanthraquinone). In both ligands phenolic ionizations occur with pK_a values near 10.0 and 13.7 as determined spectrophotometrically. Combined spectrophotometric and potentiometric analysis was used to work out the complete microconstant deprotonation scheme for daunorubicin. In daunorubicin the ammonium group deprotonates with pK_a = 8.6 and is not involved directly in metal ion binding. Spectrophotometric analysis were used to calculate stability constants. For metal ion binding to daunorubicin the order of decreasing stability constants (with stability constant logarithm in parentheses) is given by $Fe^{3+}(11.0) > Th^{4+}(10.3) > Tb^{3+}(7.2) > Zn^{2+}(4.5) > Mg^{2+}(3.7) > Ca^{2+}(3.3)$. The identical order and similar constants were found for quinizarin. For both ligands the stability constant logarithms are approximately half the literature values for tiron. Both quinizarin and daunorubicin may bind a second metal ion. One phenolic proton is displaced for each metal ion bound. Because it is partially stacked in aqueous solutions, the binding of a second daunorubicin ligand to a metal ion is more favorable than the binding of the first.

Introduction

Daunorubicin (daunomycin) and doxorubicin (adriamycin) are closely similar anthracycline antibiotics with an amino sugar. As shown in Fig. 1, the structures differ in only one ring substituent. Both antibiotics display high cytotoxicity against both normal and neoplastic cells and are in clinical use. They may interfere with DNA dependent RNA synthesis by binding to DNA [1]. Metal ions affect the interaction of these antibiotics with DNA [2]. There are only a few published reports on the interaction of metal ions with the antibiotics in the absence of DNA. An early report noted the effect of metal ions on the visible absorption spectrum [3]. A more recent investigation attempted determination of some stability constants by potentiometric titration [4].

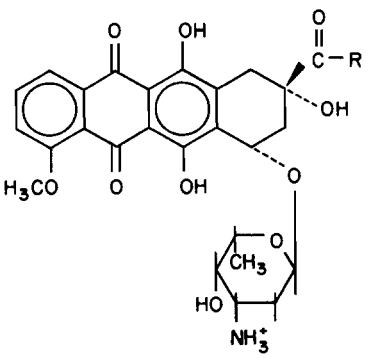

Fig. 1. Structures of daunorubicin (R = CH_3) and doxorubicin (R = CH_2OH). Quinizarin (1,4-dihydroxyanthraquinone) consists of the three leftmost rings without the methoxy group

The chromophore in daunorubicin and doxorubicin is closely related to quinizarin (1,4-dihydroxyanthraquinone) [5]. Because it is likely that this chromophore constitutes the main metal ion binding site, spectrophotometric analysis becomes especially appropriate. In this paper we report parallel studies on the equilibrium proton and metal ion binding properties of daunorubicin and quinizarin. Due to solubility limitations, the quinizarin experiments were conducted in 50% by volume ethanol while those with daunorubicin were performed in aqueous solutions.

Experimental

Daunorubicin, doxorubicin, and quinizarin were obtained from Sigma Chemical Co. Experiments for the last two compounds were performed in water and, due to insolubility, quinizarin results were obtained in 50% by volume ethanol. For both solvent systems the temperature was about 20 °C and the ionic strength about 0.15 *M*. Concentrations were standardized in stock solutions using log ϵ = 3.93 at 480 nm for quinizarin in pure ethanol [6] and ϵ = 11500 at 477 nm for daunorubicin in water [1]. Titrations were performed on a Radiometer TTT80

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Titrator and absorption spectra taken on a Cary 14R spectrophotometer. The pH meter was standardized on an activity scale with buffer solutions. Independent titrations of each metal ion in the absence of ligand reveal that hydroxo complex formation does not occur under conditions of this study. For the spectrophotometric studies a 0.05 *M* imidazole 0.05 ammonia buffer was used, except for Zn^{2+}, Fe^{3+}, and Th^{4+} where no buffers were employed. Due to small pH variations, the results for Zn^{2+} are the least accurate. Since addition of borate produces a color change, it is not suitable for a buffer.

Both quinizarin and daunorubicin are sensitive to light and oxygen [7], especially in basic solutions. Stock solutions of both compounds were stored in the dark: that of quinizarin in ethanol at room temperature and that of daunomycin in water at 5 °C. When kept at room temperature solutions of quinizarin in 50% ethanol in light lose absorption slowly at neutral pH; the rate of color loss increases to a maximum near pH 10 with a half-life of 2–3 hours and decreases to pH 12, where the rate again increases. When coordinated to metal ions the quinizarin appears more stable. Daunorubicin is less stable than quinizarin, decomposing more rapidly as the pH increases. All spectra recorded at pH > 10 were extrapolated to zero time. The absorption spectra of both ligands appears relatively stable in acid solutions.

All equilibrium constants were obtained by a nonlinear least squares analysis of the most error prone observable versus an independent variable and parameters to be determined. For spectrophotometric titrations, potentiometric titrations, and stability constants, the observables are absorbance, pH, and absorbance, respectively, while the independent variables are pH, equivalents of base added, and metal ion concentration at constant ligand concentration. The standard equations employed were derived by use of conservation of mass and equilibrium constant expressions. The ± values or values in parentheses refer to one standard deviation.

Results and Discussion

Deprotonation of quinizarin produces large changes in the visible absorption spectrum. Between $7 < pH < 11$ there is a tight isobestic point at 501 nm. Independent analysis of 15 points at each of the three wavelengths, 458, 548, and 580 nm, yields $pK_1 = 9.92 \pm 0.01$. Spectrophotometric analysis of 9 points at 564 and 602 nm yields $pK_2 = 13.7 \pm 0.1$. Potentiometric titration of the first deprotonation from quinizarin yields $pK_1 = 9.93 \pm 0.02$ in excellent agreement with the spectrophotometric value. Since there are two structurally identical acidic groups, the intrinsic (statistically corrected) acidity constants are $pk_1 = 10.22$ and $pk_{12} = 13.4$.

Deprotonation of daunorubicin also produces large changes in the visible absorption spectrum, similar to quinizarin. Unlike quinizarin, however, there is not a tight isobestic point and the pH dependence of the daunorubicin intensities does not fit a simple deprotonation. Potentiometric titration of 2 m*M* daunorubicin reveals two acidic groups, one with $pK_1 = 8.61 \pm 0.01$ and a second with $pK_2 \sim 10$. Instability of daunorubicin precludes accurate potentiometric titration of pK_2. Most of the absorption changes occur in the pK_2 region so that pK_1 may be assigned predominantly but not exclusively to the ammonium group deprotonation and pK_2 to a phenolic deprotonation.

The complete microconstant deprotonation scheme for daunorubicin appears in Fig. 2. Because the ammonium group is remote from both phenolic groups, we cannot distinguish between them, and all 2 and 3 subscripts referring to phenolic groups in Fig. 2 are interchangeable. This symmetry also occurs for the two carboxylic acid groups in the microconstant scheme for citric acid [8]. Between $9 < pH < 11.5$ the absorption spectra of daunorubicin is sensitive to deprotonation of a single phenolic group. In this pH region the fraction, α, of daunorubicin with a single deprotonated phenolic group is given by

$$\alpha = \frac{[+-0] + [+0-] + [0-0] + [00-]}{N + [+00] + [000]}$$

where N represents the 4 concentrations in the numerator. Substitution of the appropriate expressions for the microconstants yields

$$\alpha = \frac{2(k_2(H^+) + k_1 k_{12})}{2(k_2(H^+) + k_1 k_{12}) + (H^+)^2 + k_1(H^+)}$$

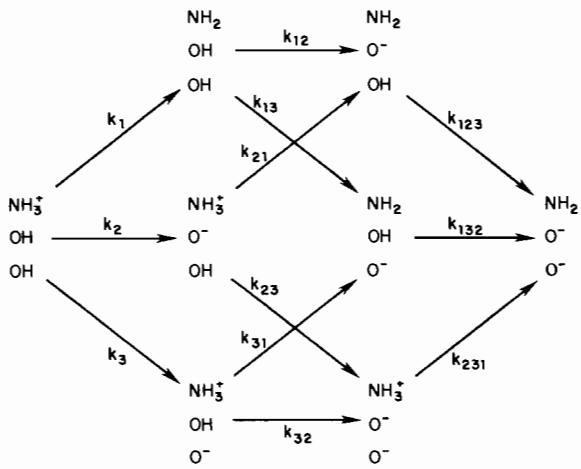

Fig. 2 Daunorubicin deprotonation scheme. The last group undergoing deprotonation is indicated by the last subscript on the acidity microconstants Subscript 1 refers to the ammonium group deprotonation and subscripts 2 and 3 to phenolic deprotonations.

Because of the relative values of the microconstants, in spectrophotometric analysis that follows k_1 cannot be accurately determined. A value of $pk_1 = 8.61$ estimated from potentiometric titration is used and later refined.

At a given wavelength the observed molar absorptivity may be expressed as

$$\epsilon = \epsilon_0(1 - \alpha) + \epsilon_2\alpha$$

where ϵ_0 refers to species with phenolic groups and ϵ_2 to species with a single phenolate group. The last equation was fitted by non-linear least squares to 14 experimental intensities from $8 < pH < 11.5$ with 0.05 m*M* daunorubicin at both 552 and 590 nm. For the sum $pk_1 + pk_{12}$ the calculated values are 19.00 ± 0.01 and 18.96 ± 0.01 at 552 and 590 nm, respectively. The best fit of the intensities at both wavelengths yields $pk_2 = 9.87 \pm 0.09$.

From the definitions of the acidity constants, the macroconstant determined by potentiometric titration is, $K_1 = k_1 + k_2 + k_3$. Only k_1 is unknown and solving yields $pk_1 = 8.66$. From the constants already evaluated, the properties of a cyclic system allow evaluation of $pk_{12} = pk_{13} = 10.32$ and $pk_{21} = pk_{13} = 9.11$. These values along with others in the microconstant scheme are collected in Table I.

TABLE I. Daunorubicin Acidity Constants.[a]

Constant	Value
pK_1	8.61
pK_2	10.0
pK_3	13.7
pk_1	8 66
pk_2, pk_3	9.87
pk_{12}, pk_{13}	10.32
pk_{21}, pk_{31}	9 11
pk_{123}, pk_{132}	13 4
pk_{23}, pk_{32}	12.95
pk_{231}	9.56

[a]In aqueous solutions at 20 °C and 0.15 *M* ionic strength.

Daunorubicin is unstable in basic solutions. To determine the macroconstant pK_3 for the second phenolic group deprotonation intensities at 590 nm were extrapolated to zero time for mixing of solutions. By this procedure we obtain $pK_3 = 13.7 \pm 0.1$. Since $K_3^{-1} = k_{123}^{-1} + k_{132}^{-1} + k_{231}^{-1}$ and the last microconstant makes a negligible contribution, $pk_{123} = pk_{132} = 13.4$.

From the microconstants evaluated so far the reciprocal effects [9] of a charged ammonium group on a phenolic ionization given by $pk_{12} - pk_2 = pk_{13} - pk_3 = 0.45$ is identical to the effect of a charged phenolate group on the ammonium deprotonation given by $pk_{21} - pk_1$. Extension of this result to other deprotonations yields $0.45 = pk_{123} - pk_{23} = pk_{132} - pk_{32} = pk_{231} - pk_{21}$. These equations are used to calculate the remaining microconstants, the values of which conform to properties of a cyclic system. All 12 microconstants of Fig. 2 for daunorubicin have been estimated and their values recorded in Table I.

The 0.45 log unit interaction between the ammonium and phenolic groups in daunorubicin is consistent with their separation by 8 and 9 bonds. The same number of bonds also separate the charge centers in pimelic and suberic acids [10] and in 1,8-diaminoctane [11]. The difference between their intrinsic acidity constants ranges from 0.28 to 0.34 log units. A significantly greater free rotation in the three long chain compounds than in the more restricted daunorubicin accounts for the reduced difference.

When the symmetry elements in the microconstant scheme for daunorubicin in Fig. 2 are included, $pk_2 - 9.3 = 9.57$ for both phenolic ionization pathways from the cation and $pk_{12} - 0.3 = 10.0$ for both pathways from the neutral molecule. At all pH values the ratio of neutral (000) to dipolar ion (+ − 0) and (+0−) species is given by $k_1/2k_2 = 8$. From the definitions of microconstants, $pk_{12} - 0.3$ is also effectively the predicted daunorubicin potentiometric macroconstat, $pK_2 = 10.0$. This value is used for comparison purposes for evaluation of the stability constants, K_M. To a good approximation in daunorubicin and doxorubicin; the ammonium group deprotonates with $pK_1 = 8.6$, the first phenolic group with $pK_2 = 10.0$, and the second phenolic group with $pK_3 = 13.7$. The last two values agree closely with the two phenolic ionizations from quinizarin.

The acidity microconstant results on daunorubicin are in good semi-quantitative agreement with several studies [12, 13]. More precise comparison is difficult because their method of calculation is restricted to a few pH values, and statistical effects do not appear to have been considered. A $pK_a = 2.1$ has been reported from potentiometric titration of 1–10 m*M* doxorubicin [4]. We find that addition of up to 1 *M* acid to either doxorubicin or daunorubicin yields little or no visible and ultraviolet spectral changes. These results are consistent with an earlier spectrophotometric result of $pK_a = -5.9$ for protonation of a carbonyl group on doxorubicin with only a single cationic species present from $-4 < pH < 7$ [12]. If there is a protonation of doxorubicin with $pK_a = 2.1$, it does not occur on the chromophoric ring system. The metal ion stability constants in reference 4 also do not agree with ours.

Metal Ion Binding

Stability constants were evaluated by a non-linear least squares fit of the observed absorption intensity as a function of total metal ion and ligand concentra-

TABLE II. Stability Constants for Quinizarin and Daunorubicin[a]

Metal Ion	Quinizarin[b]			Daunorubicin[c]		
	pH	log K_{x1}	log K_M	pH	log K_{x1}	log K_M
H^+						8 6
			9.92			10 0
			13 7			13 7
Mg^{2+}	7 92	2.09(1)	4.09	8.29	2 01(1)	3.73
	8.49	2 67(1)	4.12	8 55	2 25(2)	3 72
				8 89	2.60(1)	3 74
				9.26	3 01(4)	3 82
Ca^{2+}	8.50	1.76(3)	3.20	8 97	2 16(8)	3.23
	9 11	2.29(2)	3 16	9 13	2 46(9)	3 38
				9 51	2.94(9)	3.55
Tb^{3+}	5.04	2.2(1)	7.1	5 59	2 78(1)	7.19
	5.93	3 10(1)	7 09	6.13	3 42(2)	7 29
	6 50	3.60(3)	7.02	7 07	4 3(2)	7.25
Zn^{2+}	6.40	1 39(4)	4 9	6.40	0.8	4.4
	6 55	1.42(8)	4.8	6 60	1 1	4.5
Fe^{3+}	0.46	1.43(2)	10.89	0.53	1.63(2)	11.10
	1 02	2 09(3)	10 99	0 70	1 69(3)	10 99
Th^{4+}	1.07	1 33(1)	10 18	0.94	1 19(11)	10.25
	1.40	1.74(1)	10 26	1.28	1.57(5)	10 29

[a]At 20 °C and 0.15 *M* ionic strength. [b]In 50% ethanol. [c]In aqueous solutions.

tions and the parameters to be fitted: stability constants and absorption intensities of specified complexes. Addition of metal ions increases the absorption in the 560 nm region of quinizarin. In a set of experiments the pH and total ligand concentration were held constant and the metal ion concentration increased from equimolar to about a 100 fold excess. Values for the calculated equilibrium constant, $K_{x1} = [MLH]/([M](C_L - [MLH]))$, where C_L is the total ligand concentration, for the reactions $M + HLH \rightleftarrows MLH + H^+$ are tabulated in Table II. Values of the usual stability constant for $M + LH \rightleftarrows MLH$ are given by $[MLH]/([M][LH]) = K_M = K_{x1}(1 + (H^+)/K_a)$, where $pK_a = 9.92$ are also listed in Table II. The good agreement between log K_M values for each metal ion indicates that metal ion binding displaces one proton.

Metal ion binding to daunorubicin increases the absorption intensity in the 575 nm region. A five to several hundred fold metal ion excess was used to produce sufficient intensity increases for evaluation of stability constants. Values of the equilibrium constant, K_{x1}, and the stability constant, K_M, as described above ($pK_a = 10.0$), appear in Table II. There is no observable complex formation at pH 1 between $Hg(ClO_4)_2$ and quinizarin, tiron, and the chromophore of daunorubicin.

In addition to the 1:1 complexes so far described, for which stability constants are listed in Table II, 2:1 and 1:2 metal ion to ligand complexes also form under some conditions. We define the equilibrium constant $K_3 = [MLM]/([M][MLH])$ for formation of MLM according to $MLH + M \rightleftarrows MLM + H^+$. Binding of Tb^{3+} to quinizarin is so strong at pH 5.93 that formation of a 2·1 complex MLM becomes significant in solutions containing up to 400 fold excess Tb^{3+}. The results were refined by noting that absorption at 572 nm is more sensitive to M_2L and absorption at 560 nm more sensitive to MLH. At pH 5.93 we find that log $K_{x1} = 3.10$ and log $K_3 = 2.3$. The 560 nm absorption for the latter complex is about 50% stronger than that for the former.

Several daunorubicin solutions contained sufficient amounts of MLM at high metal ion to ligand ratios to permit an estimate of their log K_3 stability constants for MLM. At pH 9.26 binding of up to 10^3 excess Mg^{2+} yields measurable quantities of the MLM complex. Addition of Mg^{2+} decreases the intensity at 470 nm and produces an intensity maximum at 578 nm. At both wavelengths log $K_{x1} = 3.01$ as listed in Table II. Also we determine at both wavelengths at pH 9.26, log $K_3 = 1.9 \pm 0.1$. Similarly we find for Ca^{2+} at pH 9.51, log $K_3 = 1.5 \pm 0.2$. Tb^{3+} binds more strongly than Mg^{2+} and Ca^{2+}, and it was possible to evaluate K_3 at two pH values. For Tb^{3+} we deduce at pH 5.59, log $K_3 = 1.7 \pm 0.1$, and at pH 6.13, log $K_3 = 2.2 \pm 0.2$. For daunorubicin, with all three metals ions the pH-dependent log K_3 values are 1.1–1.4 log units less than the log K_{x1} values at the same pH.

An interesting contrast occurs between quinizarin and daunorubicin $M(LH)_2$ complexes prevalent in the presence of excess ligand. Analysis by non-linear least squares of the absorption intensity at 560 nm for 13 solutions containing 0.07–1.5 moles Tb^{3+} per mole of quinizarin at pH 6.50 yields log K_{x1} = 3.60 and log K_{x2} = 2.8 for addition of a second ligand according to $MLH + HLH \rightleftarrows M(LH)_2 + H^+$. The log K_{x1} value yields a log K_M value in good agreement with values calculated from a metal ion excess at pH 5.04 and pH 5.93 (Table II). For quinizarin and Tb^{3+} in 50% ethanol the difference log K_{x1} – log K_{x2} = 0.8, a typical difference for first and second stability constants.

Results were obtained for daunorubicin and Tb^{3+} at pH 7.07 at 0.1 metal ion equivalent intervals up to 1 equiv and at integral equiv to 10 equiv to test for formation of $M(LH)_2$. For formation of MLH, log K_{x1} = 4.3, while log K_{x2} = 5.3 for addition of a second ligand. The log K_{x1} value yields a log K_M value in close agreement with the values calculated for pH 5.59 and pH 6.13 with a large metal ion excess. That complexation of the second ligand of daunorubicin exhibits a 10 fold greater stability conconstant than the first may be ascribed to daunorubicin stacking which is known to occur in aqueous solutions of the free ligand [14]. For quinizarin in 50% ethanol little or no stacking occurs, and formation of $M(LH)_2$ is not similarly favored. Considering both ligands together, the stacking increases log K_{x2} for daunorubicin by 1.8 log units. For equimolar solutions of daunorubicin and metal ion in which more than 10% of the ligand is complexed, there is more daunorubicin present in the $M(LH)_2$ than in the MLH complex.

Almost all studies were conducted at 25 or 50 u*M* daunorubicin, where most of the molecules are not stacked. Experiments were performed with excess Fe^{3+} at pH 0.70 at both 0.049 and 0.100 m*M* daunorubicin. At the higher concentration about 50% of the daunorubicin is stacked if a dimerization constant of 10 mM^{-1} is assumed [14]. The results do not refine well in absence of a dimer species. In a non-linear least squares analysis of 27 experiments, the species considered were HLM, $(HLH)_2$, and MLH. A good refinement gives log K_{x1} = 1.69(3) and K_D = 10.1(3) mM^{-1}. The latter result for the daunorubicin dimerization constant derived indirectly from intensities of the Fe^{3+} complex is in remarkable agreement with the result obtained independently from solutions containing the free ligand at pH 7.0 [14]. The superb agreement between values of the dimerization constant determined at pH 0.7 and at pH 7 provides an additional argument against protonation of ligand with a pK_a = 2.1.

The pH range of the Fe^{3+} experiments is limited by metal ion hydrolysis at pH > 1 and by complex ion formation with anions at pH < 0.5. Both quinizarin and daunorubicin bind Fe^{3+} so weakly at pH < 1 that metal ion hydrolysis begins before MLM complex formation. Daunorubicin or doxorubicin cannot bind three Fe^{3+} ions at pH 7 to form the proposed simple complex triferric doxorubicin (quelamycin) used in some cancer therapy [15].

For both the quinizarin and daunorubicin results in Table II the stability constant K_M for the reaction $M + LH \rightleftarrows MLH$ remains nearly constant for each metal ion. This result establishes that binding of each metal ion displaces one proton. This result also applies to the MLM complexes described above where the 1.1–1.4 lesser log values for daunorubicin indicate that the second metal ion bound also displaces a single proton.

There is a strong similarity between the log K_M values in Table II for quinizarin and daunorubicin for each metal ion. For both ligands the order of decreasing stability constants (with quinizarin stability constant logarithms in parentheses) is Fe^{3+} (10.9) > Th^{4+} (10.2) > Tb^{3+} (7.1) > Zn^{2+} (4.9) > Mg^{2+} (4.1) > Ca^{2+} (3.2). This similarity and the related spectral changes indicate that metal ion binding to daunorubicin (and doxorubicin) occurs primarily at the anthracycline chromophore. Little or no binding occurs at the amino group. Deprotonation of the protonated amino group from 8 < pH < 9 may account for the small upward drift in daunorubicin log K_M values in Table 2 for Mg^{2+} and Ca^{2+}. For both of these metal ions the experiments were performed through the region of ammonium group deprotonation. The effect is expected to be comparable to the 0.45 log units interaction found above for the phenolic–ammonium group protonations. From the stability constants it is possible to show that in body fluids only small fractions of daunorubicin and doxorubicin bear a Mg^{2+} or Ca^{2+} ion.

Under conditions similar to those of this investigation, the first stability constant log K_M values for the first 5 metal ions of Table II and tiron (1,2-dihydroxybenzene-3,5-disulfonate) are 6.9, 5.8, 14.1, 10.3, and 20.4, respectively [16]. Plots of the log K_M values for quinizarin and daunorubicin from Table II *versus* those for tiron yield straight lines that pass near the origin with slope 0.52 ± 0.03. In quinizarin and daunorubicin metal ion chelation occurs at a phenolic site with $pK_a \cong 10.0$ while in tiron chelation occurs at a catecholate function with a sum $pK_1 + pK_2 = 7.7 + 12.6 = 20.3$. The points for hydronium ion fall nearly on the straight lines found for the 5 metal ions. The slope near 0.5 is due to the quinizarin and daunorubicin pK_a values being almost exactly half the tiron $pK_1 + pK_2$ sum. This correlation may be put to practical use. For those metal ions for which the stability constant log K_M for binding to daunorubicin or doxorubicin is unknown, this constant may be estimated simply by taking half of a value recorded for tiron.

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