Self-Association of Doxorubicin and Related Compounds in Aqueous Solution

MILENA MENOZZI ˣ, LUIGI VALENTINI, ELISABETTA VANNINI, and FEDERICO ARCAMONE

Received January 24, 1983, from *Farmitalia Carlo Erba, Ricerca e Sviluppo Chimico, Via dei Gracchi 35, 20146 Milano, Italy.* Accepted for publication May 11, 1983.

Abstract □ The dimerization of doxorubicin, daunorubicin, and their 4-demethoxy, 4′-epi, and 4′-deoxy analogues was studied spectrophotometrically. Self-association was found to be influenced by buffer composition and ionic strength. K_d values were 1.3×10^4 and 2.3×10^4 M^{-1} for doxorubicin and daunorubicin, respectively, and ranged from 3.8×10^3 to 6.1×10^3 M^{-1} for the 4-demethoxy analogues. For 4′-epi- and 4′-deoxydoxorubicin, tetramerization has also been considered. On this basis, values of 2.0×10^4 and 2.2×10^4 M^{-1} were found, respectively, for the formation constant of the dimerization process. Stability of the dimeric species appears to be strongly influenced by substitution of the chromophore moiety.

Keyphrases □ Doxorubicin—self-association, 4-demethoxy, 4′-epi, 4′-deoxy, 4-demethoxy-4′-epi, analogues, effect of buffer concentration and ionic strength □ Daunorubicin—self-association, 4-demethoxy analogues, effect of buffer concentration and ionic strength □ Dimerization—doxorubicin, daunorubicin, and their 4-demethoxy, 4′-epi, and 4′-deoxy analogues, effect of buffer concentration and ionic strength

During investigations carried out in this laboratory on doxorubicin–biopolymer interactions, knowledge of the drug dimerization process under the conditions used was necessary. The self-association of doxorubicin (I) and daunorubicin (II) in aqueous solutions has been the subject of several studies; such self-association was generally considered to be of the same type as that shown by actinomycin (1), acridine dyes (2, 3), and purine derivatives (4). The cooperative-type phenomena related to the effect of ligand dimerization on the manifestation of the binding process have been well documented (3, 5, 6).

For this reason, and also because the available literature data (7–15) exhibited large variations apparently related to the different experimental conditions used, we have determined the dimerization constants of these clinically important anthracyclines in three aqueous buffer systems, employing a spectrophotometric technique with the elimination of interference due to fluorescence. Figure 1 shows the differences among specific absorption spectra of doxorubicin at various concentrations in Tris buffer plus NaCl. These differences are considered to be due to dimerization (8, 10, 12). New second generation antitumor anthracyclines, showing more favorable pharmacological properties, have also been included in the study.

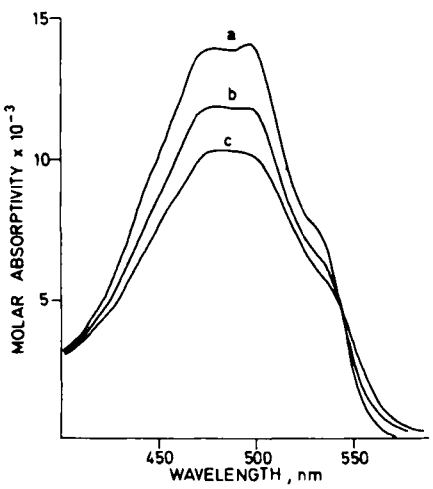
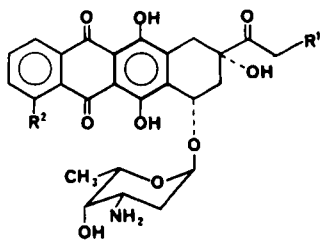
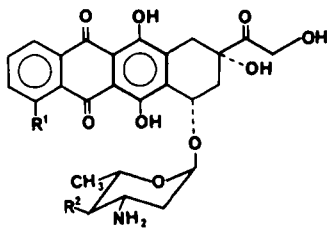


Figure 1—*Visible absorption spectra of I at different concentrations in pH = 7.0 Tris buffer + NaCl. Key: (a) 1.7×10^{-6} M; (b) 1.7×10^{-5} M; (c) 1.7×10^{-4} M.*



I : R^1 = OH ; R^2 = OCH_3
II : R^1 = H ; R^2 = OCH_3
III : R^1 = OH ; R^2 = H
IV : R^1 = R^2 = H



V : R^1 = OCH_3 ; R^2 = OH
VI : R^1 = OCH_3 ; R^2 = H
VII : R^1 = H ; R^2 = OH

EXPERIMENTAL

Chemicals—Doxorubicin (I), daunorubicin (II), 4-demethoxydoxorubicin (III), 4-demethoxydaunorubicin (IV), 4′-epidoxorubicin (V), 4′-deoxydoxorubicin (VI), and 4-demethoxy-4′-epidoxorubicin (VII) were used as hydrochlorides. All the drugs were isolated or synthesized in our laboratories and purified by crystallization as hydrochloride salts. The chemical titers of I–VII were ≥97% as determined by an HPLC procedure (16). Phosphate and Tris buffer solutions at pH 7.0 were prepared at the same ionic strength (μ = 0.05). Tris buffer plus NaCl was prepared by adding NaCl (final concentration: 0.15 M) to pH 7.0, 0.05 M Tris-HCl in order to make the ionic strength 0.196 M.

Apparatus—Spectrophotometric measurements[1] were carried out at 477 nm (22°C), and interferential filters were placed beyond the cells to cut off the emitted fluorescence. The concentration range of the drugs was 1×10^{-6}–5×10^{-4} M, and 5-, 2-, and 1-cm path length cells were used. The wall adsorption effect, as already described by others (11), prevents the use of cells of small volume (such as 0.1-cm path length) for which the surface to volume ratio is very unfavorable.

[1] Coleman, Perkin-Elmer model 575.

0022-3549/84/0600-0766$01.00/0

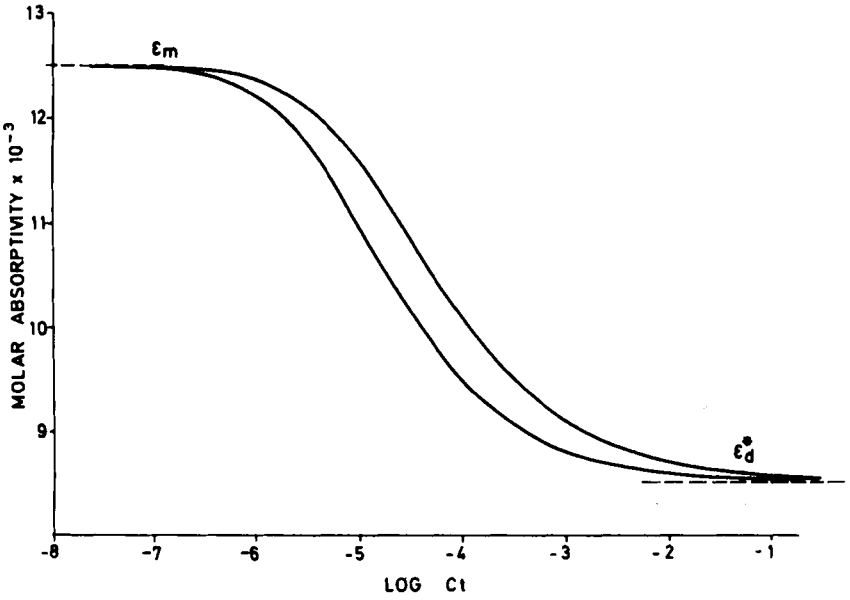


Figure 2—*Theoretical behavior of* $A/[C_t] = f([C_t])$ *(upper) and* $dA/d[C_t] = f([C_t])$ *(lower), described by Eqs. 5 and 6, respectively.*

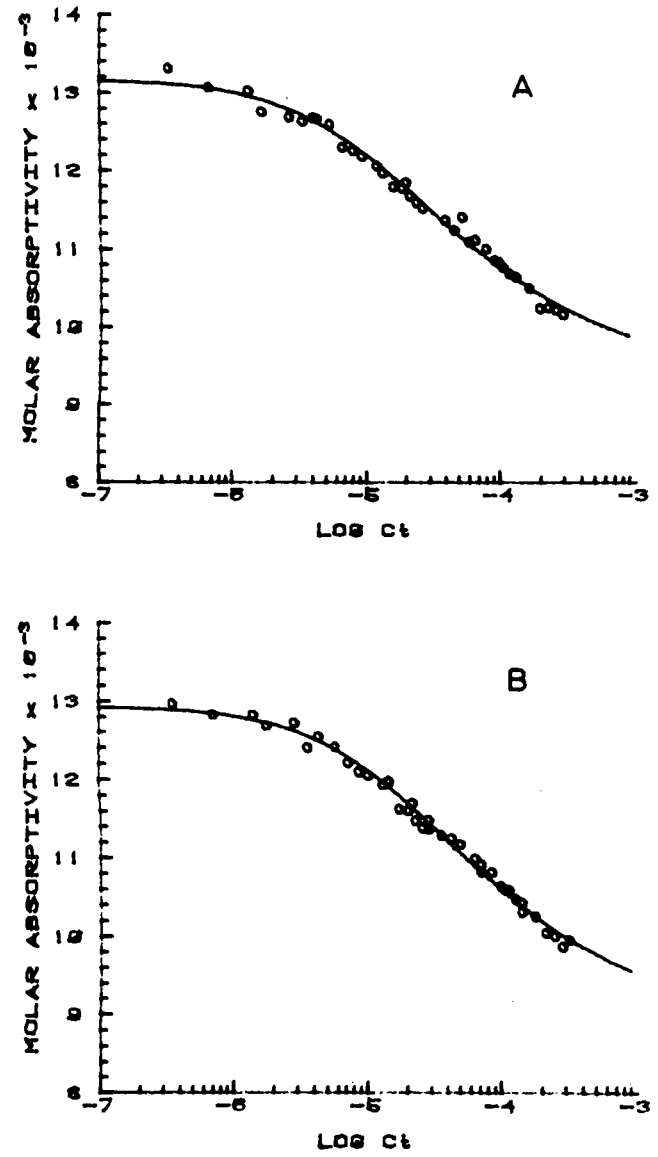


Figure 3—*Molar absorptivity* versus *log* C_t *for I (A) and II (B) in pH 7.0 Tris buffer + NaCl, according to the direct procedure. The solid lines represent the best fit of experimental points according to Eq. 5.*

Direct Procedure—Different amounts of a stock drug aqueous solution were diluted with the buffer up to the desired concentrations. The absorbance of each sample was measured without delay in 5- and 1-cm path length cells according to the concentration used. Each sample was prepared at least in triplicate.

Incremental Procedure—Increasing amounts of buffered stock drug solution were pipetted into the cell containing a predetermined volume of the same buffer. For concentration values ranging from 8×10^{-7} to 3×10^{-5} M, 15-mL cells of 2-cm path length were employed, with a starting volume of 10 mL of buffer solution, to which from 200 to 900 μL of buffered stock drug solution was added. For concentration values ranging from 6×10^{-6} to 2×10^{-4} M, 1-cm path length cells containing 2 mL of buffer were used. Absorbance was measured after the addition of increasing amounts (from 50 to 200 μL) of the drug solution.

True Spectra of Monomer and Dimer—The true spectra of monomer and dimer were obtained by differential measurements, using two cuvettes of different path length (5 and 1 cm) in order to allow subtraction of the absorption contribution due to the other species.

THEORETICAL

The absorbance of a monomeric species in equilibrium with its dimeric form is defined by:

$$A = \epsilon_m[C_m] + 2\epsilon_d^*[C_d] \qquad \text{(Eq. 1)}$$

where A is the experimental absorbance divided by the cell path length (in centimeters) at a stated wavelength; ϵ_m and ϵ_d^* are the molar absorptivities of monomer and dimer, respectively, calculated using the monomer molecular weight; and $[C_m]$ and $[C_d]$ are the molar concentrations of the monomer and dimer, respectively. For the mass balance, the total concentration is:

$$[C_t] = [C_m] + 2[C_d] \qquad \text{(Eq. 2)}$$

where $[C_t]$ is the total molar concentration expressed as monomeric units. The dimerization constant is defined by:

$$K_d = \frac{[C_d]}{[C_m]^2} \qquad \text{(Eq. 3)}$$

Table I—Dimerization Constants and Molar Absorptivities

Compound	$K_d \pm SE$, $10^3 \times M^{-1}$	$\epsilon_m \pm SE$, $10^3 \times M^{-1} \times cm^{-1}$	$\epsilon_d^* \pm SE$, $10^3 \times M^{-1} \times cm^{-1}$
I	16.64 ± 2.16[a]	13.10 ± 0.05	9.26 ± 0.14
	12.99 ± 1.19[b]	13.15 ± 0.03	9.27 ± 0.09
	22.79 ± 2.68[c]	13.16 ± 0.05	9.33 ± 0.09
	17.18 ± 2.34[c,d]	13.08 ± 0.08	8.15 ± 0.15
II	12.98 ± 1.74[a]	12.96 ± 0.06	8.84 ± 0.14
	13.44 ± 1.91[b]	12.93 ± 0.05	8.85 ± 0.15
	16.07 ± 1.53[c]	12.93 ± 0.04	8.88 ± 0.09

[a] pH 7.0, μ = 0.05, phosphate buffer. [b] pH 7.0, μ = 0.05, Tris buffer. [c] pH 7.0, μ = 0.196, Tris buffer + NaCl. [d] Value obtained without interferential filters.

Table II—Dimerization and Tetramerization Constants

Compound	$K_d \pm SE$, $10^3 \times M^{-1}$	$\epsilon_m \pm SE$, $10^3 \times M^{-1} \times cm^{-1}$	$\epsilon_d^* \pm SE$, $10^3 \times M^{-1} \times cm^{-1}$	$K_t \pm SE$, $10^3 \times M^{-1}$	$\epsilon_t^* \pm SE$, $10^3 \times M^{-1} \times cm^{-1}$
V[a]	11.10 ± 0.76	13.05 ± 0.03	8.18 ± 0.09		
	19.84 ± 12.28[c]	13.10 ± 0.05	9.57 ± 1.59	9.61 ± 3.01	8.43 ± 0.49
VI[a]	12.54 ± 0.94	13.07 ± 0.04	7.72 ± 0.10		
	22.13 ± 11.94[c]	13.14 ± 0.05	9.11 ± 1.44	6.84 ± 3.64	7.68 ± 0.97
III[a]	6.11 ± 1.01	10.79 ± 0.01	7.64 ± 0.33		
IV[a]	4.25 ± 0.57	10.79 ± 0.03	6.79 ± 0.20		
VII[b]	3.79 ± 1.40	10.46 ± 0.07	6.66 ± 0.60		

[a] pH 7.0, μ = 0.196, Tris buffer + NaCl. [b] pH 7.0, μ = 0.05, Tris buffer. [c] The large value of SE is due to the high correlation coefficients found between the parameters of the model.

Equations 1–3 can provide the evaluation of the dimerization constant (5, 11). Solving the system of Eqs. 1–3 for the absorbance in terms of the total concentration, one obtains:

$$A = \epsilon_d^*[C_t] + \frac{\Delta\epsilon}{4K_d}\sqrt{1 + 8K_d[C_t]} - \frac{\Delta\epsilon}{4K_d} \qquad \text{(Eq. 4)}$$

where $\Delta\epsilon = \epsilon_m - \epsilon_d^*$. Dividing Eq. 4 by $[C_t]$ the result is:

$$\frac{A}{[C_t]} = \epsilon_d^* + \frac{\Delta\epsilon}{4K_d[C_t]}(\sqrt{1 + 8K_d[C_t]} - 1) \qquad \text{(Eq. 5)}$$

and by derivation of Eq. 4 with respect to $[C_t]$ we obtain:

$$\frac{dA}{d[C_t]} = \frac{\Delta\epsilon}{\sqrt{1 + 8K_d[C_t]}} + \epsilon_d^* \qquad \text{(Eq. 6)}$$

The unknown parameters of Eqs. 4–6 are K_d, ϵ_m, and ϵ_d^*. They can be simultaneously estimated by a nonlinear least-squares fitting procedure from each of the three preceeding equations. Theoretical behavior of functions 5 and 6 is shown in Fig. 2. Data obtained following the direct procedure were treated by Eq. 5, applying a modified version of the Fletcher and Shrager program (17). The incremental procedure was employed for I and II in phosphate buffer. The data obtained, together with those from the direct procedure, were simultaneously treated by a nonlinear fitting, taking into account both Eqs. 5 and 6. The parameters describing the tetramerization process were obtained by the same fitting procedure, employing a suitable extension of Eqs. 1–3 to obtain the solving equation corresponding to Eq. 5.

RESULTS

Doxorubicin and Daunorubicin—Figure 3 shows the molar absorptivities of I and II as a function of the total concentration in pH 7.0 Tris buffer plus NaCl, μ = 0.196, following the direct procedure. Estimates of K_d, ϵ_m, and ϵ_d^*, obtained from the best fitting of Eq. 5 for the three buffers employed, are reported in Table I. Different K_d values were found for the three conditions.

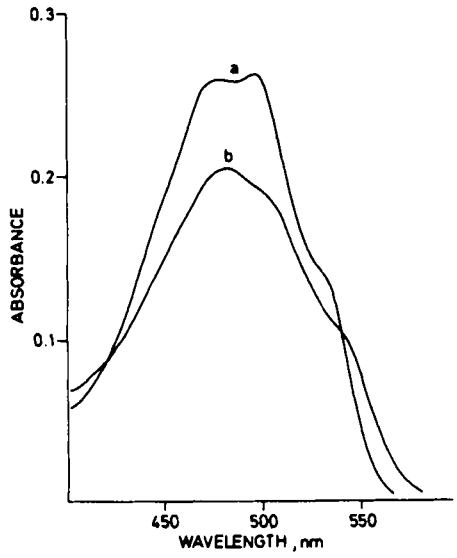


Figure 4—*True spectra of I monomer (a) and dimer (b), obtained by differential measurements, at 2.14 × 10⁻⁵ M × cm in pH 7.0 Tris buffer + NaCl. Key: (a) 1.05 × 10⁻⁵ M solution in a 5-cm cuvette against 3.10 × 10⁻⁵ M solution in a 1-cm cuvette; (b) 5.10 × 10⁻⁵ M solution in a 1-cm cuvette against 5.92 × 10⁻⁶ M solution in a 5-cm cuvette.*

Figure 4 shows the differential spectra, in Tris buffer plus NaCl, of the monomeric and dimeric species of I. The concentrations were calculated using the corresponding parameters reported in Table I.

Figure 5 reports the molar absorptivities obtained from the direct and incremental procedure for I and II in phosphate buffer. The two sets of experimental data were treated simultaneously as described above. The values of the parameters K_d, ϵ_m, and ϵ_d^* so obtained were found to be in close agreement with those presented in Table I. To evaluate the influence of the ionic strength on K_d values, measurements were performed in pH 7.0 phosphate buffer, with concentrations ranging from ~1.8×10^{-3} to 1.8×10^{-1} M. K_d values were

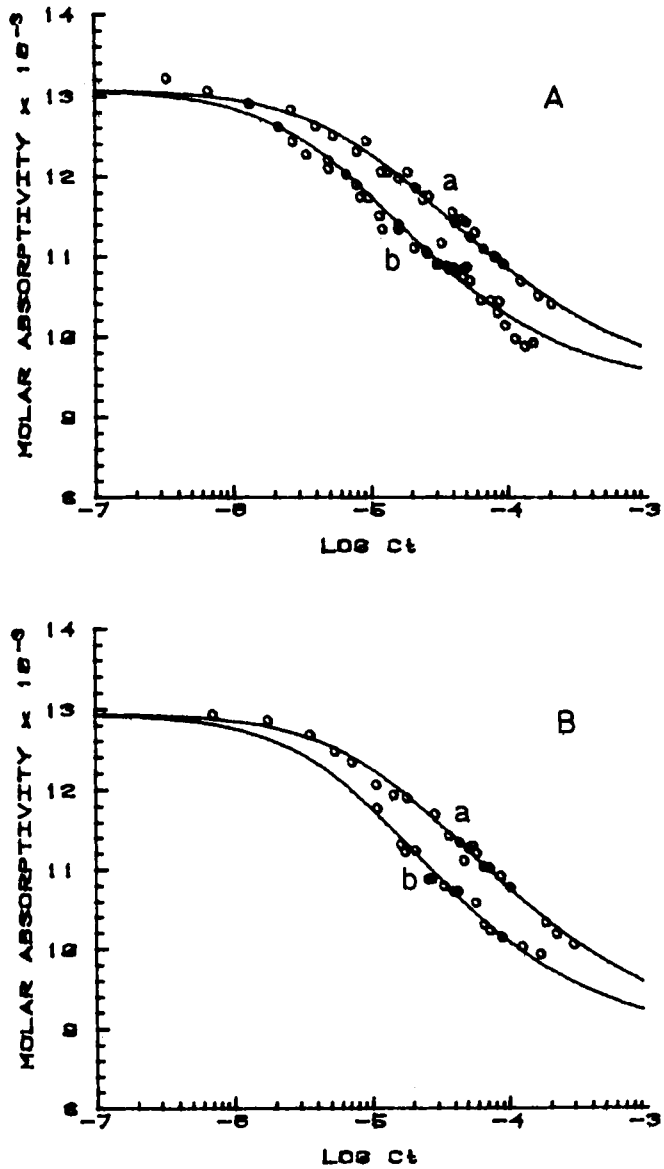


Figure 5—*Molar absorptivity (a) and incremental molar absorptivity (b) versus log C_t for I (A) and II (B), in pH 7.0 phosphate buffer, according to the direct and incremental procedures.*

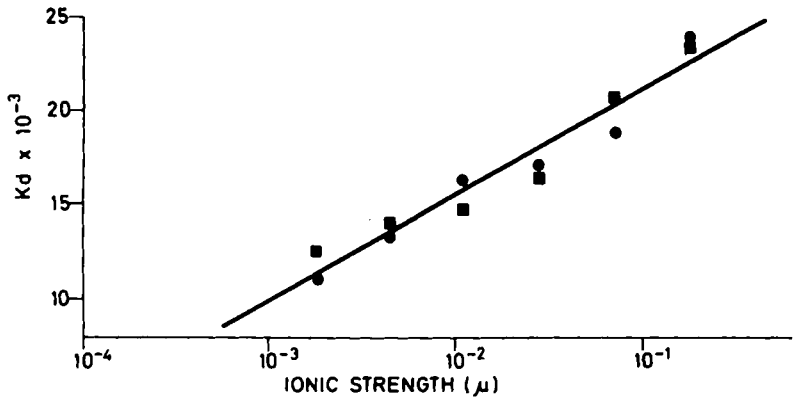


Figure 6—*Variation of the* K_d *as a function of the ionic strength.*

obtained from the absorbance measurements applying Eqs. 1-3 and using the ϵ_m and ϵ_d^* values previously determined. The dependence of K_d on the ionic strength is reported in Fig. 6.

4'-Epi, 4'-Deoxy, and 4-Demethoxy Analogues—As shown in Table II, V and VI exhibit apparent ϵ_d^* and K_d values significantly lower than those of the parent drug. The corresponding dimerization curves are presented in Fig. 7. Table II also shows the results obtained for V and VI when a tetramerization process is taken into account. The occurrence of such a process is strongly suggested by the behavior of the curves of Fig. 7 and by the low value of ϵ_d^* calculated on the basis of a simple dimerization.

A clearly lower dimerization constant is also shown by the analogues carrying a modification in the chromophore portion of the molecule, namely the 4-demethoxy derivatives (III, IV, and VII). Figure 8 shows the dimerization curves of the latter compounds.

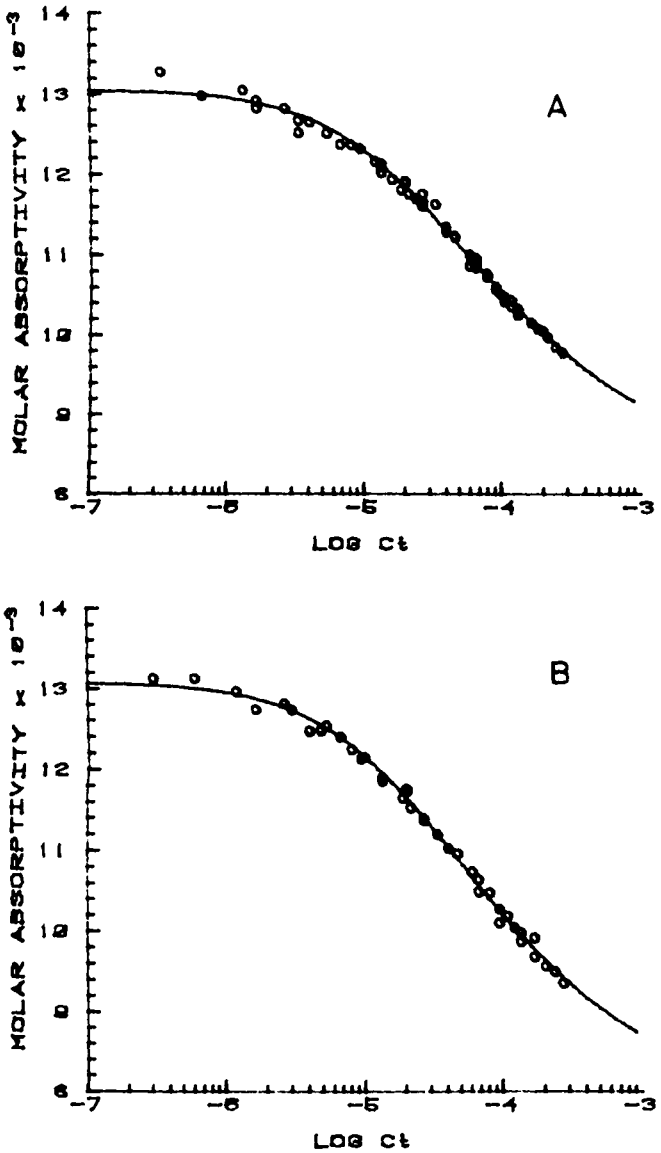


Figure 7—*Molar absorptivity* versus *log* C_t *for V (A) and VI (B). The conditions were the same as those in Fig. 3.*

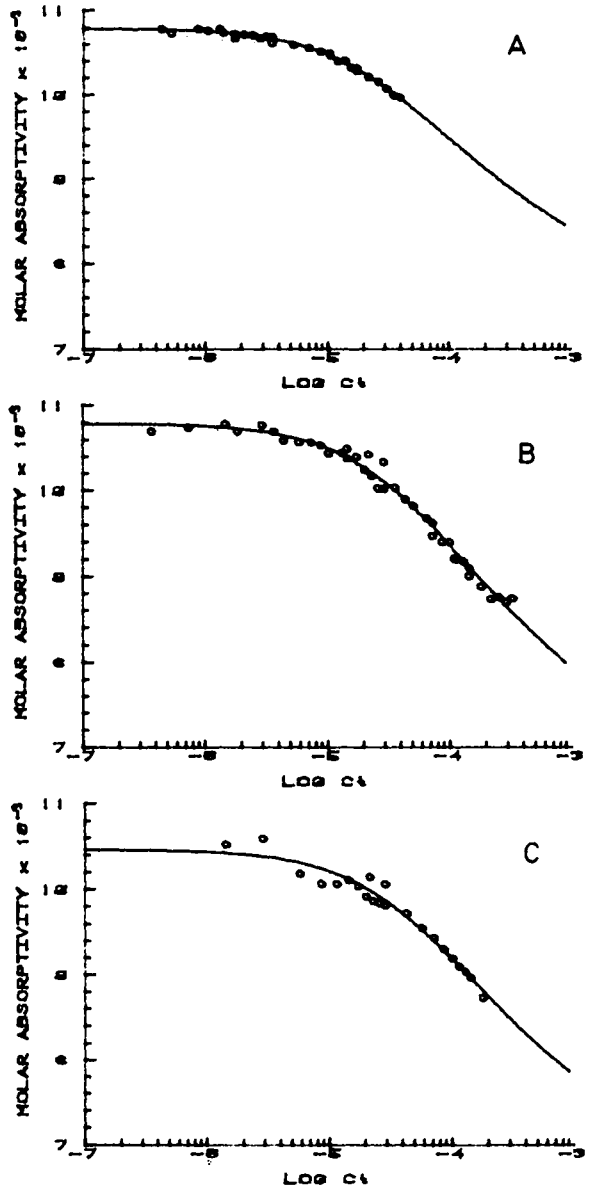


Figure 8—*Molar absorptivity* versus *log* C_t *for III (A), IV (B), and VII (C). Compound VII was in 0.05 M Tris buffer; all other conditions were the same as those in Fig. 3.*

DISCUSSION

The spectrophotometric behavior of I and II, as shown by the curves of Figs. 3 and 5, is near to that theoretically expected for a simple dimerization process. The values of the parameters obtained in different conditions (Table I) indicate that the dimerization constant is dependent on both the buffer composition and ionic strength. The K_d values observed for I seem to experience, on increase of the ionic strength, a larger variation than the corresponding K_d values of II, strongly suggesting an involvement of the 14-hydroxyl group in the dimerization process, either directly (*e.g.*, by formation of hydrogen bonds) or indirectly (*e.g.*, dipole effects). The dimerization constant values of I and II, as found in this study, are generally larger than those reported by others. Indeed the K_d values we found are twice as large as those reported by Stutter *et al.* (14) and Martin (12) for I and II, respectively, although the latter author worked with a lower buffer ionic strength. The results we obtained are practically identical to those reported by Eksborg (9), but markedly differ from those of Schütz *et al.* (11) and Chaires *et al.* (15) for II. No explanation can be given at this point for these differences, and it may be noted that the values of the molar extinction coefficients of both monomer and dimer given by these authors are not in agreement with the values reported in the present study.

Among the hundreds of doxorubicin analogues, V and VI have been selected for further development toward clinical evaluation because of their favorable pharmacological properties (18, 19). In this connection, a comparison of their properties at the molecular level with those of I is of particular interest. As regard to self-aggregation in solution, the two analogues either give rise to a somewhat less stable dimeric species or show a higher tendency toward the formation of tetramers. In both cases the latter interpretation of the results would give a slightly better fitting of the experimental data (RMS[2] = 70.2 for V and 75.8 for VI) than does the former (RMS = 75 and 81 for V and VI, respectively). The greater ability to form tetrameric species can be tentatively related to the lower hydrophilicity of V and VI as compared with I, favoring hydrophobic stacking aggregation. Accepting the tetramerization hypothesis, the dimerization constants for V and VI are very near to that found for I, in agreement with the identical electronic structure of the chromophoric moiety.

A clearly different situation exists in the case of the 4-demethoxy analogues. Compounds III, IV, and VII show a distinctly lower tendency toward dimerization when compared with the parent drugs. The reduced dimer stability appears to be a consequence of the substitution of the C-4 methoxyl group with a hydrogen atom, a modification that strongly influences the electronic structure of the chromophore. The demethoxy analogues are up to 10 times more potent than the corresponding methoxylated derivatives in different biological systems (20, 21); this property is not based on a higher affinity for the DNA receptor (22). Whether the lower tendency to self-aggregation mirrors a diminished tendency to other aspecific interactions and therefore increases, for instance, the rate of accumulation within the cells and correspondingly the availability at the receptor site, remains to be established.

[2] Root mean square.

REFERENCES

(1) N. S. Angerman, T. A. Victor, C. L. Bell, and S. S. Danyluk, *Biochemistry*, **11**, 2402 (1972).

(2) H. H. Li and D. M. Crothers, *Biopolymers*, **8**, 217 (1969).

(3) W. Müller and D. M. Crothers, *Eur. J. Biochem.*, **54**, 267 (1975).

(4) J. L. Dimicoli and C. Hélène, *J. Am. Chem. Soc.*, **95**, 1036 (1973).

(5) G. Schwarz, S. Klose, and W. Balthasar, *Eur. J. Biochem.*, **12**, 454 (1970).

(6) G. Schwarz and W. Balthasar, *Eur. J. Biochem.*, **12**, 461 (1970).

(7) V. Barthelemy-Clavey, J. L. Maurizot, J. L. Dimicoli, and P. Sicard, *FEBS Lett.*, **46**, 5 (1974).

(8) V. Crescenzi and F. Quadrifoglio, in "Polyelectrolytes and Their Applications," A. Rembaum and E. Sélégny, Eds., D. Reidel Publishing Dordrecht, Holland, 1975, p. 217.

(9) S. Eksborg, *J. Pharm. Sci.*, **67**, 782 (1978).

(10) H. Porumb, *Prog. Biophys. Mol. Biol.*, **34**, 175 (1978).

(11) H. Schütz, F. A. Gollmick, and E. Stutter, *Stud. Biophys.*, **75**, 147 (1979).

(12) S. R. Martin, *Biopolymers*, **19**, 713 (1980).

(13) E. Goormaghtigh, P. Chatelain, J. Caspers, and J. M. Ruysschaert, *Biochim. Biophys. Acta*, **597**, 1 (1980).

(14) E. Stutter, F. A. Gollmick, and H. Schütz, *Stud. Biophys.*, **88**, 131 (1982).

(15) J. B. Chaires, N. Dattagupta, and D. M. Crothers, *Biochemistry*, **21**, 3927 (1982).

(16) "Code of Federal Regulations," Food and Drug Administration, Washington, D.C., April 1, 1982, § 436.322.

(17) J. E. Fletcher and R. I. Shrager, "A User's Guide to Least-Squares Model Fitting," DCRT Technical Report No. 1, 1968.

(18) A. M. Casazza, A. Di Marco, C. Bertazzoli, F. Formelli, F. Giuliani, and G. Pratesi, in "Current Chemotherapy," vol. 2, W. Siegenthaler and R. Luthy, Eds., American Society for Microbiology, Washington, D.C., 1978, p. 1257.

(19) F. Arcamone, S. Penco, S. Redaelli, and S. Hanessian, *J. Med. Chem.*, **19**, 1424 (1976).

(20) A. M. Casazza, G. Pratesi, F. Giuliani, and A. Di Marco, *Tumori*, **66**, 549 (1980).

(21) A. Di Marco, A. M. Casazza, F. Giuliani, G. Pratesi, F. Arcamone, L. Bernardi, P. Giardino, and B. Patelli, *Cancer Treat. Rep.*, **62**, 375 (1978).

(22) F. Arcamone, "Doxorubicin Anticancer Antibiotics: Medicinal Chemistry," vol. 17, Academic, New York, N.Y., 1981, p. 265.

ACKNOWLEDGMENTS

The authors are very indebted to Prof. A. Vacca, University of Florence, for his helpful contribution in data handling.