PROTOLYTIC EQUILIBRIA OF DOXORUBICIN AS DETERMINED BY ISOELECTRIC FOCUSING AND 'ELECTROPHORETIC TITRATION CURVES'

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1. Introduction

Doxorubicin (Adriamycin^R) and the related compound daunorubicin (Daunomycin) are antibiotics of the anthracycline group [1] with cytotoxic and antimitotic activity [2]. Their clinical uses in the treatment of acute leukemia and solid tumors have been reviewed [3–5]. Their biological activity is linked to the formation of an intercalative complex with DNA [6–8] with concomitant inhibition of both RNA and DNA synthesis [9,10]. Doxorubicin is also known to interact with phospholipids [11,12] and with sulphated mucopolysaccharides [13]. On the other hand, Daunorubicin has been shown to bind to non-histone protein from rat liver [14] and, due to the presence of planar aromatic rings in its quinone moiety, to interact strongly with itself [15], forming mostly dimers and tetramers, as already shown for many similar compounds like actinomycin, proflavin, purines or acridine orange. This self-association, in turn, should affect the acid dissociation constants of the two groups ionizable at or near physiological pH, i.e., the 3'-NH_2 group in the sugar moiety and the 11-OH group in the quinone ring. This could explain why literature data on the amino group pK values vary from 7.2–8.99 [1,16,17] and from pK 9.54–10.1 for the phenolic –OH [16,18]. These pK' values have been determined by potentiometric (amino group) as well as by spectrophotometric and fluorimetric (phenolic –OH) titrations and, more recently, on the basis of partition coefficients in chloroform–1-pentanol [16].

In this report, we have attempted to measure protolytic equilibria of doxorubicin indirectly, by equilibrium isoelectric focusing, by pI and ΔpI value determinations, and directly, by using 'electrophoretic titration curves'. This last technique, described [19,20], has been successfully applied to the study of macromolecule–ligands [21] and macromolecule–macromolecule interactions [22].

2. Materials and methods

Unlabelled and [^{14}C]doxorubicin (spec. act. 24.5 μCi/mg) were prepared as hydrochloride derivatives at the Farmitalia Laboratories. Ampholine (pH 3.5–10) was from LKB Produkter AB, Bromma. Urea (ultrapure), acrylamide and *N,N'*-methylene bisacrylamide were from Bio Rad Labs, Richmond, CA.

Equilibrium isoelectric focusing [23], the two dimensional isoelectric focusing–electrophoresis technique [20] and pH measurements [24] were performed as described. In 8 M urea gels, pH measurements were made by eluting the gel slices in 300 μl 10 mM KCl–8 M urea solution and then by subtracting from the pH readings a correction factor of 0.42 pH units [25]. When focusing [^{14}C]doxorubicin, 2 mm gel segments were cut along the separation track, transferred to counting vials, and eluted overnight in 0.5 ml 10 mM KCl. After additon of 8 ml Packard Insta Gel (Downers Grove, IL) the samples were counted in a Beckman LS 3100 β-spectrometer. pH measurements were made on 2 mm gel strips cut alongside the gel strips used for radioisotope counting.

The spectrophotometric titration curves were

performed with a Coleman Perkin Elmer 575 in 0.04% ampholine buffers covering the pH 5–11 range. This procedure was found to give more reproducible results than titrations in standard buffers.

3. Results and discussion

Figure 1 shows the [^{14}C]doxorubicin profile in isoelectric focusing as a function of progressive dilution (from 6–0.2 μg/ml). The insert shows focused unlabelled doxorubicin at higher concentrations (from 100 μg/ml to 7 mg/ml). It can be seen that doxorubicin is indeed an amphoteric molecule, as predictable from its structure, and is also a 'good carrier ampholyte' [26] since it focuses very sharply (pI – pK difference <1.0). Moreover, the isoelectric point (pI) is seen to be a function of the concentration, and progressively decreases with increasing dilutions, from pI 9.33 (>2 mg/ml) down to pI 8.76 ($\leqslant$1 μg/ml). These pI variations must reflect pK alterations of ionizable groups in doxorubicin brought about by self-association.

In fig.2 we have plotted pI values as a function of concentration. On the diluted side, pI constancy (pI 8.76) is reached in ~2 μM solutions, while on the concentrated side a pI plateau (pI 9.33) is attained in ~2 mM solutions. This means that these concentrations must correspond to fully disaggregated (monomeric, ~μM solutions) and fully aggregated (tetrameric or n-meric species, ~mM solutions), respectively. When these experiments are repeated in 8 M urea, the monomeric pI value is kept at much higher concentrations, $\leqslant$100 μg/ml (~0.2 mM solutions) indicating that urea effectively disaggregates these complexes. At higher concentrations, the pI rises but not above

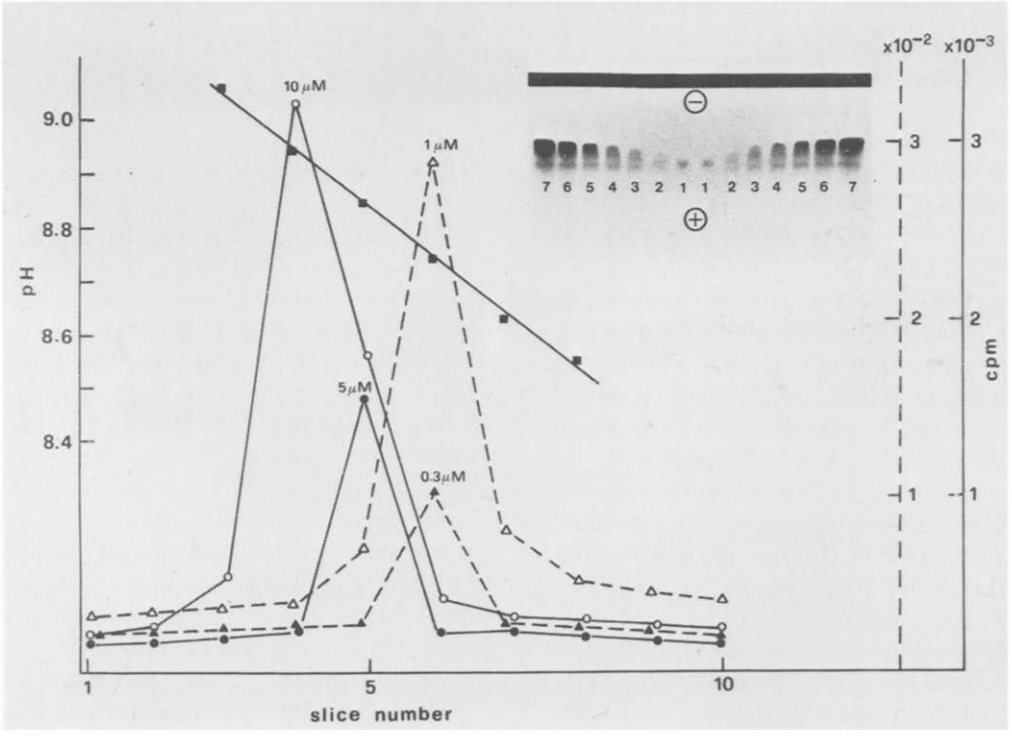

Fig.1. Isoelectric focusing of [^{14}C]doxorubicin in a 7% acrylamide gel, containing 2% Ampholine (pH 3.5–10). The sample was applied in pockets precast in the gel at the anodic side. After focusing, 2 mm gel segments were cut along the separation track and counted as in section 2. Solid lines refer to the 3000 cpm full scale, while broken lines refer to the 300 cpm scale. (■ – ■) pH gradient. Insert: focusing of unlabelled doxorubicin at >100 μg/ml. Loads: (1) 0.1 mg/ml; (2) 0.2 mg/ml; (3) 0.5 mg/ml; (4) 1 mg/ml; (5) 2.5 mg/ml; (6) 5 mg/ml; (7) 7.5 mg/ml. Of each stock solution, 20 μl were loaded in the gel. Notice the bow-shaped profile, indicating a decreasing of pI with progressive sample dilution. The black strip is the electrode filter paper.

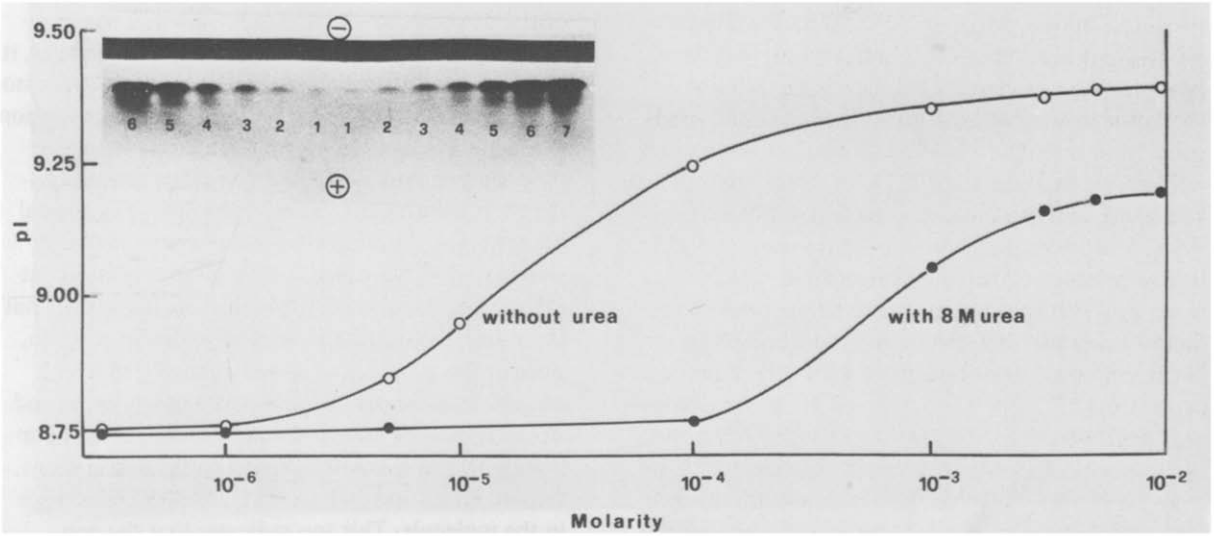

Fig.2. Variation of the pI of doxorubicin as a function of its concentration in solution upon focusing in the presence and in the absence of urea. The plateau in the diluted side indicates the presence of (mostly) monomeric species while the plateau in the concentrated side indicates the attainment of fully aggregated forms. Notice that in 8 M urea the monomeric state is kept at 100-fold higher concentration (~0.2 mM) than in the absence of urea. The insert (same samples and loads as in fig.1) shows the focusing of unlabelled doxorubicin in an 8 M urea gel. Notice the much gentler arching of the samples, as compared to fig.1, indicating the attainment of an aggregation state of lower degree.

a limiting pI 9.2, indicating that the aggregate forms do not reach the polymeric order attainable in absence of urea. This is in agreement with our studies with dyes [27], where full disaggregation at high concentrations could only be achieved by combining 8 M urea with high temperatures (55°C) and with the data in [15], where the circular dicroic spectrum of concentrated daunorubicin was shown to be shifted at high temperatures toward the spectrum of diluted solutions.

These data do not allow us to calculate any of the two pK values of doxorubicin. However, since the pI must be equidistant from the two pK values, one of the pK values can be easily derived from the well known equation $pI = (pK_1 + pK_2)/2$ provided the other pK is measured by an independent method. Since the sugar amino group is several atoms removed from the aromatic chromophore, its degree of protonation should not affect the electronic spectrum of doxorubicin, therefore the spectrophotometric titration of the phenolic group should give an accurate estimate of its pK. Figure 3 reports the pK value of the phenolic group and its variation upon dilution. Since in ~2 mM solutions the pK_{-OH} 10.15, a $pK_{-NH_3^+}$ 8.51 is derived. In very dilute solutions (~2 μM), pK_{-OH} 9.60, from which a $pK_{-NH_3^+}$ 7.92 can be calculated. Thus, monomeric doxorubicin should have a

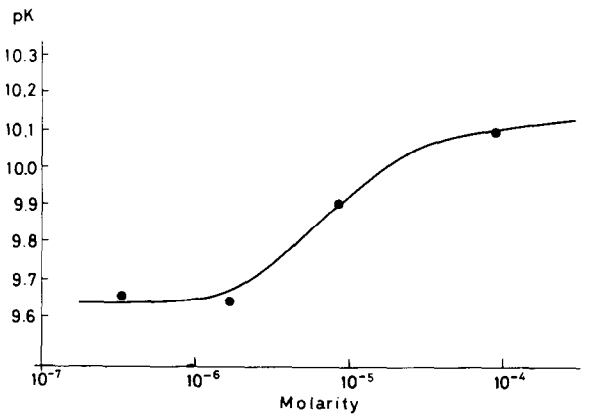

Fig.3. Spectrophotometric titration curve of the phenolic –OH. Titrations were performed in 0.04% Ampholine (pH 5–11) with a Coleman Perkin Elmer 575 spectrophotometer. At each concentration analyzed, the pK of the phenolic –OH has been graphically derived as the inflection point of the titration curve obtained by plotting the A_{553} values as a function of pH.

pK_{-OH} 9.60 and $pK_{-NH_3^+}$ 7.92. While the phenolic group pK is in excellent agreement with [16], the $pK_{-NH_3^+}$ 7.2 ± 0.05 reported therein seems to be much too low in comparison with our pK value and other literature data [13–15]. If this low value were correct, we should find pI 8.37 for dilute doxorubicin solutions, while we cannot reach a pI <8.76 even in 0.3 μM solutions, i.e., with dilutions lower than [15] or comparable to [16] literature values.

In fig.4, we have attempted to titrate directly the amino group by the recently described technique of 'electrophoretic titration curves' [20–23]. The shape

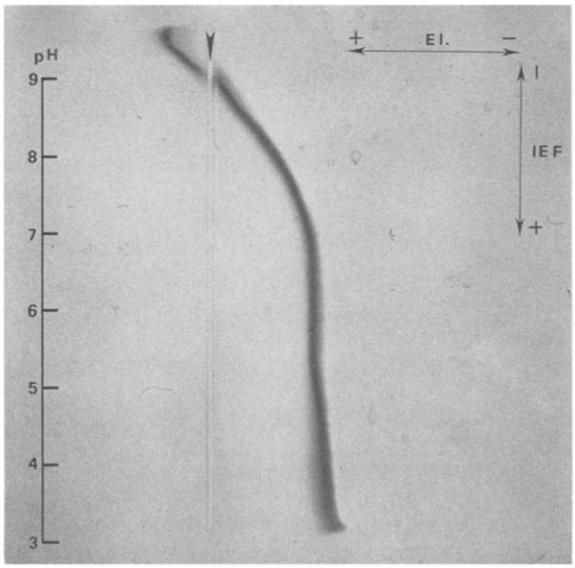

Fig.4. Direct 'electrophoretic titration curve' of doxorubicin. Gel slab (2 mm thick): 7% acrylamide, 2% Ampholine (pH 3.5–10), 5 mM Asp, 5 mM Glu, 5 mM Lys and 8 M urea. First dimension: 90 min run in the LKB Multiphor chamber by delivering 10 W with an LKB constant wattage power supply. Coolant temperature: 10°C. Second dimension: 15 min run by delivering 600 V (constant) across 13 cm electrode distance. The two arrows and positive and negative symbols represent the direction and polarity of isoelectric focusing (IEF) and electrophoresis (El.). The arrowhead indicates the sample application zone (i.e. the zero-mobility or isoelectric plane). The two black strips are the electrolyte filter paper strips used in the second dimension. The pH gradient shown is already corrected for the presence of 8 M urea. The solution loaded here is doxorubicin at 0.5 mg/ml but the pK calculations have been performed on an identical run with 0.1 mg/ml (which did not show any deviation from ideal behavior), not shown because too faint for a good photographic reproduction.

of the doxorubicin curve clearly shows that, as the molecule moves against the stationary pH gradient, it is continuously titrated over pH 3–10. Above the isoelectric point (the pH of crossing over the application trench) it behaves in fact as an anion, changing to the bright violet color of the phenolate ion. Below the pI, it behaves as a cation, reverting to its typical orange color. As the amino group is progressively protonated, the cationic mobility increases until, at pH ~7, constant mobility is achieved, indicating that the amino group is fully charged (or very nearly so, since at this point small charge changes can go undetected). At pH <7 all the way down to the end of the titration curve, the doxorubicin band is completely flat, and exactly parallel to the application trench, indicating that no charge changes take place in the molecule. This too indicates that the app. pK 7.2 reported [16] cannot hold true, otherwise constant mobility would have been achieved at pH ~6, rather than pH ~7. The apparent mobility increase at the acidic end of the titration curve is an artefact, probably due to an edge effect, or perhaps to interaction with carrier ampholytes, since this phenomenon is greatly enhanced when the titration curve is run in the absence of urea.

In order to obtain exact pK values from the titration curve, we have derived a mathematical equation describing it. By assuming that:

1. The total electrophoretic mobility (M_t) of a mono-mono- valent amphoteric molecule is proportional to its degree of ionization;
2. The viscosity and degree of polymerization of acrylamide are constant along the gel length;
3. The conductivity along each focused Ampholine species is constant;
4. The molecule does not change size or shape along the pH 3–10 gradient;

we have derived the following equation (full mathematical treatment will be given elsewhere):

$$M_t = h \frac{10^{pK_c - pH} - 10^{pH - pK_a}}{1 + 10^{pK_c - pH} + 10^{pH - pK_a}} \quad (1)$$

where K_c and K_a are the dissociation constants of cations and anions, respectively, and h is a proportionality coefficient. In our case, since accurate mobility measurements can only be made in the

'cationic' portion of the titration curve, pK_c will be derived by measuring the pH ($pH_{½}$) corresponding to ½ mobility in the cathodic direction, corrected by a correction factor accounting for the influence of the degree of ionization of the phenolate ion on the value of ½ mobility. Thus, by substituting in eq. (1) the value $pK_a = 2\ pI - pK_c$ and the value pH ½ corresponding to ½ mobility, we obtain:

$$pK_c = pH_{½} - \log (1 - 3 \times 10^{-2\ (pI - pH_{½})}$$

In our case, from the experimental values of pI 8.76 and $pH_{½}$ 7.92, a pK_c 7.95 is derived (in this case, the correction factor is very small, due to the distance between the two pK values), from which a pK_a 9.57 is automatically obtained. These values are in excellent agreement with the two pK values determined by direct titration of the phenolate ion and by equilibrium focusing. Notice that from a single titration curve the pI as well as the two pK values can be simultaneously calculated. To our knowledge, this is the first time that the use of isoelectric focusing for the direct measurement of pK values is reported.

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