SELF-ASSOCIATION OF DAUNORUBICIN

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1. Introduction

Daunorubicin is an antibiotic of the anthracycline group isolated from cultures of *Streptomyces Caeruleorubidus* with cytoxic and antimitotic activities. Presently it is used in the treatment of acute leukemia [1]. A considerable body of evidence has been accumulated which is in agreement with the existence of an intercalative complex with the DNA [2], which results in inhibition of both enzymatic RNA and DNA synthesis [3,4]. The study of the physico-chemical properties of the DNA–Daunorubicin complex [5] led us to a preliminary study of the behaviour of this molecule in solution. It could be expected that, due to its planar aromatic ring, strong interactions between molecules could exist as already shown for many compounds such as actinomycin [6], proflavine [7], purine derivatives [8–10] or acridine orange [11].

This paper reports results of a study on the effects of the antibiotic concentration investigated by various physicochemical methods: ultraviolet and visible absorption, circular dichroism (CD) and nuclear magnetic resonance (NMR). From the comparison between the results yielded by these different methods a model for the self-association of Daunorubicin is proposed.

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2. Materials and methods

Daunorubicin (also known as Rubidomycin or Daunomycin) 13057 R.P. was a generous gift of Rhône Poulenc S.A. It was in the form of hydrochloride ($C_{27}H_{29}O_{10}N$, HCl, H_2O; mol. wt. 582, 02); its formula is shown on fig. 1. All experiments were performed in 0.1 SSC (1.5×10^{-2} M NaCl; 1.5×10^{-3} sodium citrate; pH 7.0). Samples with concentration between 3.10^{-5} M and 0.35 M were stocked at 4°C in the dark.

Circular dichroism and absorption spectra were recorded with Roussel-Jouan and Cary 14 spectrophotometers respectively. Quartz cells from 0.01 mm to 100 mm were used.

Proton magnetic resonance spectra were recorded with a Bruker HFX 90 MHz spectrometer equipped with a Fabritek 1072 computer. All chemical shifts were measured with respect to an external reference

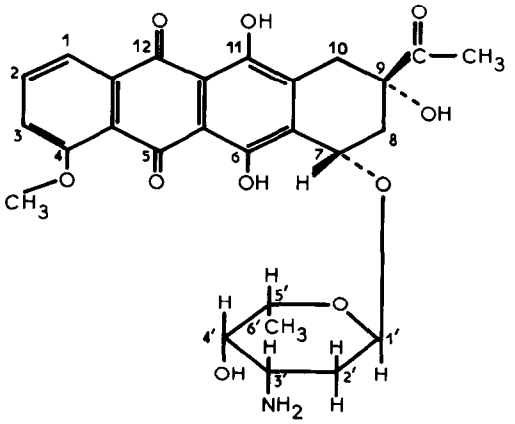

Fig. 1. Structure of Daunorubicin.

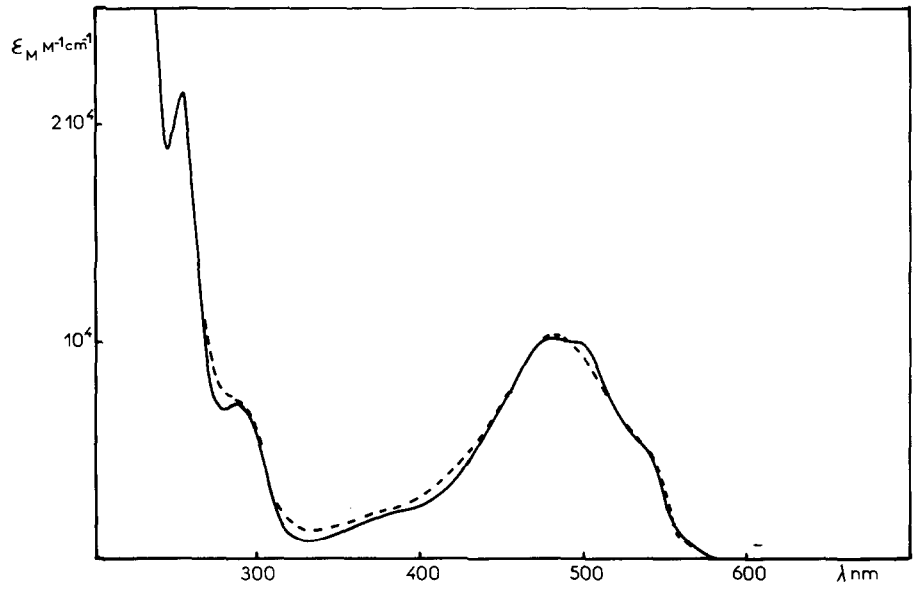

Fig. 2. Absorption spectra of Daunorubicin at two concentrations: (——) C = 3.35 10^{-5} M; (- - -) C = 1.67 10^{-3} M. Buffer is 15 mM sodium chloride, 1.5 mM sodium citrate, pH 7.0.

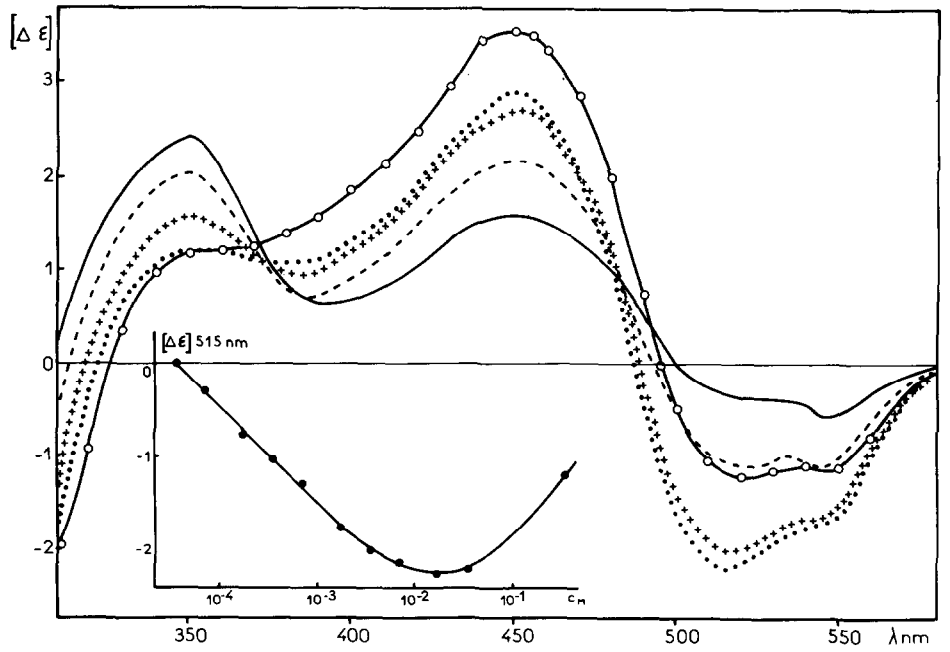

Fig. 3. Circular dichroism spectra of Daunorubicin for different concentrations. (——) C = 6.88 × 10^{-4} M; (+ + + +) C = 3.44 × 10^{-3} M; (· · · ·) C = 3.44 × 10^{-2} M; (-o-o-) C = 0.344 M. Insert: the variation of the circular dichroism intensity at 515 nm as a function of the Daunorubicin concentration.

(H.M.S.). Spectra were taken in D_2O at pD = 7 (PD = pH + 0.4). Precision on the chemical shifts are 0.1 Hz for the higher concentrations and 0.3 Hz for the smaller concentrations of Daunorubicin.

3. Results

3.1. *Absorption spectra*

The fig. 2 shows the absorption spectra of Daunorubicin at low (3.4×10^{-5}) and high (1.7×10^{-3} M) concentrations. The first spectrum agrees very well with those previously described by Bernard et al. [1]. As concentration of Daunorubicin increases there are weak but definite changes in the shape of this spectrum. The maxima located at 290 and 495 nm vanish, while the absorption increases in the wavelength range 300–400 nm. For concentration lower than 2.10^{-3} M the absorption follows the Beer–Lambert law. For higher concentrations deviations are observed; however, the very reduced thickness of the cell does not allow sufficient precision for quantitative analysis.

3.2. *Circular dichroism study*

Fig. 3 shows the CD spectra of Daunorubicin at different concentrations in the wavelength range 300–600 nm. At low concentration, when Daunorubicin is mainly in monomeric form, its spectrum is composed of two negative bands with maxima at 545 and 515 nm, and two positive bands with maxima at 350 and 450 nm. Increasing the concentration of antibiotic leads to important changes in its CD spectrum.

– The intensities of the two negative bands decrease and reach a minimum for a concentration of about 2×10^{-2} M of antibiotic. For larger concentration there is an increase of the CD signal at these two wavelengths (515 and 540 nm).

– There is an increase of the CD signal at 450 nm whereas at 350 nm there is a decrease of the intensity.

– For concentration lower than 2×10^{-2} M there are two isoelliptic points at 375 and 482 nm.

When Daunorubicin concentration increases, these changes in the CD spectrum do reflect intermolecular association between antibiotic molecules. We have tried to analyse this result in terms of an assumed equilibrium between monomeric and dimeric species. Such a model has been found to be valid for many dyes, as for exemple, acridine orange [11], or proflavine [7]. Furthermore, it was shown that Daunorubicin when crystallized is found as a dimer [12]. The existence of isoelliptic point for the lower concentration of Daunorubicin is also in agreement with this hypothesis.

If we assume that the concentration of dimeric species is low as compared with that of monomer, we can use the following relationship [7]:

$$\frac{1}{\Delta\epsilon - [\Delta\epsilon_R]} = \frac{1}{R_0 K([\Delta\epsilon_D] - 2[\Delta\epsilon_R])} + \frac{4}{[\Delta\epsilon_D] - 2[\Delta\epsilon_R]}. \quad (1)$$

$[\Delta\epsilon_R]$ and $[\Delta\epsilon_D]$ are respectively the molecular circular dichroic absorption of the monomer and dimer at a given wavelength, and $\Delta\epsilon$ is the circular dichroic absorption of the sample per molecule of daunorubicin. R_0 is the total concentration of Daunorubicin and K the association constant corresponding to the equilibrium:

$$R + R \rightleftarrows R_2.$$

$[\Delta\epsilon_R]$ is obtained by extrapolation at infinite dilution of the curve $\Delta\epsilon = f(R_0)$. We used the intensity of the CD signal at 350 nm for the calculation. Fig. 4 shows that the plot

$$\frac{1}{\Delta\epsilon - [\Delta\epsilon_R]} = f\left(\frac{1}{R_0}\right)$$

is a straight line for the lower values of the concentration, as predicted from the relationship (1). This indicates that in this range of concentration the dimerization model is valid. A value of 570 M^{-1} is obtained for K; a similar value is obtained when using the intensity at 450 nm. There are two possible explanations for the deviation from linearity observed for the higher concentrations: a) the concentration of dimer cannot be neglected as compared to that of monomer; b) for such a concentration the model of dimerization is not valid and higher association takes place. The value of 570 M^{-1} found for the association constant indicates that the first explanation is true but does not exclude the second one. However, qualitative information can be obtained from the

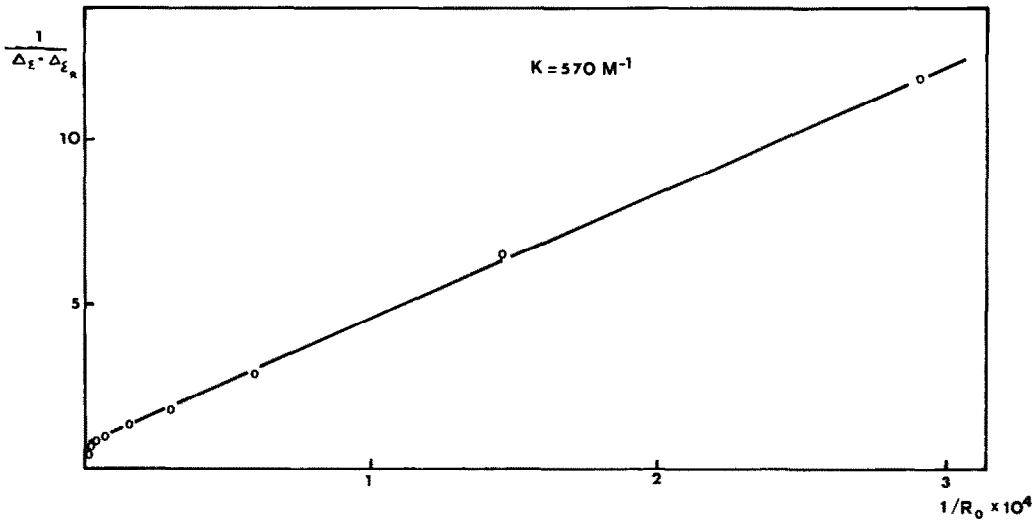

Fig. 4. Dimerization constant settled by circular dichroism data. (See text, eq. (1), for details.)

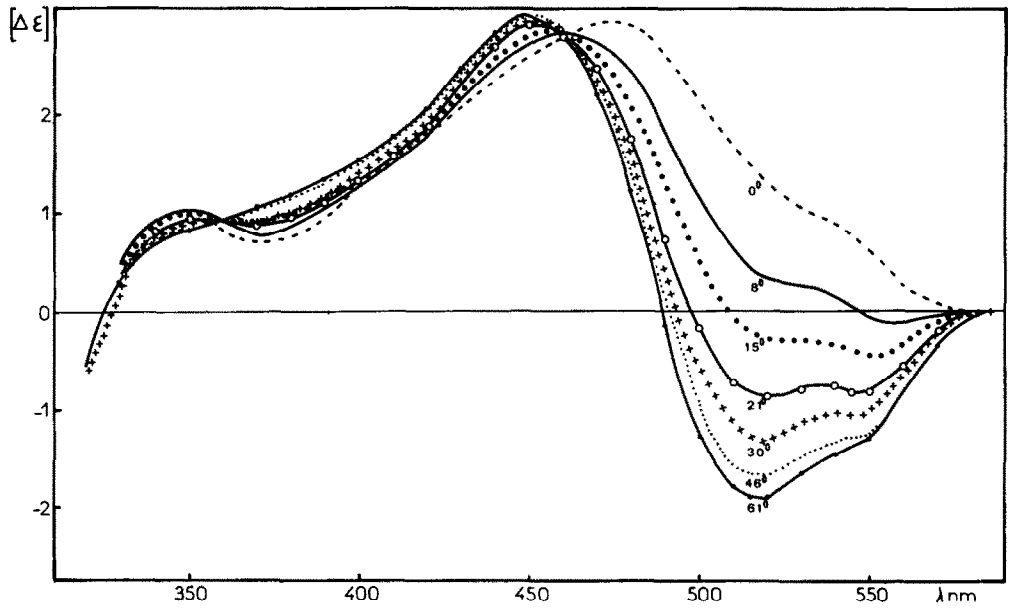

Fig. 5. Circular dichroism spectra of Daunorubicin as a function a temperature; concentration of Daunorubicin is 0.344 M.

study of temperature effect on solutions of high concentration. Fig. 5 shows that decreasing the temperature leads to a change in the CD spectrum of Daunorubicin at high concentration; the two bands with maxima at 515 and 545 nm become positive, while at 350 and 450 nm there are small variations of the intensities, which are in the contrary to those induced by the increase of antibiotic concentration. That this effect does not result of a conformational change of the Daunorubicin itself, is demonstrated by the very weak effect of the temperature on the CD spectrum of Daunorubicin at low concentration.

It can be tentatively concluded from these observations that at higher concentration there is a self-association, which gives rise to species larger than dimers and which are characterized by a CD spectrum with positive contributions between 500 and 600 nm.

3.3. *Nuclear magnetic resonance study*

We used the results of Bernard et al. [1] for the interpretation of the NMR spectra of Daunorubicin in aqueous solution. Increasing the concentration of antibiotic leads to upfield shifts of the resonance of all the protons and particularly those of the aromatic ring. Between 4×10^{-3} M and 1.5×10^{-1} M. The upfield shifts of the aromatic proton are 36 Hz (high field aromatic protons) and 33 Hz (low field aromatic protons), while it is 23 Hz for the methoxy group in similar conditions. Such shifts cannot be attributed to a change in the magnetic susceptibility which can be estimated 4 Hz for higher concentration. These observed shifts result from the self-association of the aromatic rings, whose large magnetic anisotropy induces upfield shifts of the neighbouring molecules [13].

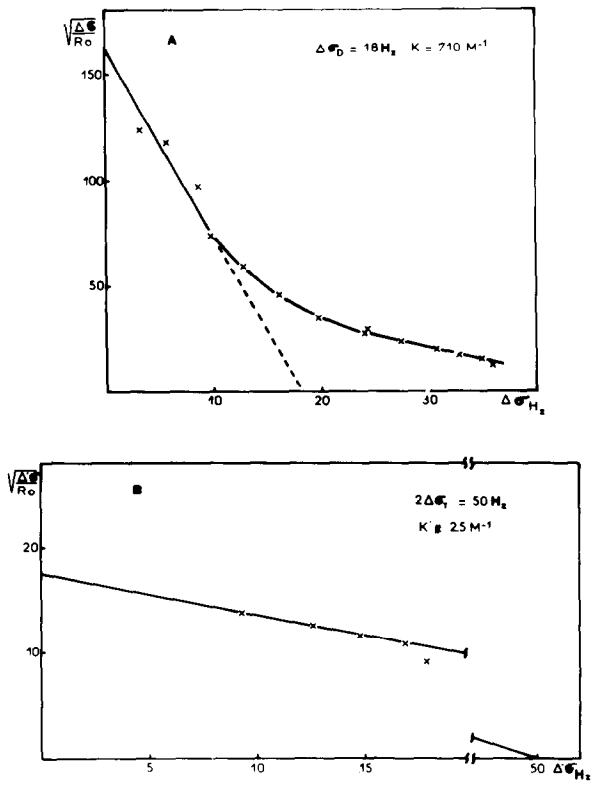

Fig. 6. A) Dimerization constant settled by NMR data at low concentrations of Daunorubicin (see text, eq. (2) for details). B) Second dimerization constant settled by NMR data at high concentrations of antibiotic. (See text, (3) for details.)

For quantitative analysis of the NMR spectra we used the signal of the methoxy group since it is easier to detect than those of the aromatic protons. We first applied the model of dimerization, which was shown valid by CD measurements, to our NMR results. The following relationship was used [10]:

$$\sqrt{\frac{\Delta\epsilon}{R_0}} = \sqrt{\frac{2K}{\Delta\sigma_D}}\ (\Delta\sigma_D - \sigma) \qquad (2)$$

where $\Delta\sigma = \sigma - \sigma_R$ and $\Delta\sigma_D = \sigma_D - \sigma_R$. σ_R, σ_D and σ are the chemical shifts of the monomer, dimer and of the sample. R_0 is the total concentration of antibiotic. A plot of $(\frac{\Delta\sigma}{R_0})^{1/2}$ against $\Delta\sigma$ will give a straight line whose slope and x axis intercept are $(\frac{2K}{\Delta\sigma_D})^{1/2}$ and $\Delta\sigma_D$ respectively. σ_R is obtained by extrapolation of the chemical shift to zero concentration. The fig. 6A shows that for the lower concentration the dimerization model seems correct. The value of the association constant K is 700 M^{-1} and $\Delta\sigma_D$ is 18 Hz. For high concentration of antibiotic, the dimerization model is not valid. Self-association forming n-mers with successive identical association constant can also be excluded. In this case the relationship (2) becomes

$$\sqrt{\frac{\Delta\sigma}{R_0}} = \sqrt{\frac{K}{2\Delta\sigma_D}}\ (2\,\Delta\sigma_D - \Delta\sigma) \qquad (3)$$

and a plot of $(\frac{\Delta\sigma}{R_0})^{1/2}$ against $\Delta\sigma$ will thus give a straight line as already calculated for dimer. (If this relationship is used for the lower concentration of Daunorubicin, the value obtained for $\Delta\sigma_D$ seems too small for self-association). The self-association of Daunorubicin at high concentration of antibiotic produces species larger than dimers, and at least two association constants are needed to describe the process. We tried to analyse the self-association assuming that at high concentration only dimers self associate, with an association constant K' eq. (2) or (3) becomes

$$\sqrt{\frac{\Delta'\sigma}{R_0}} = \sqrt{\frac{2K'}{\Delta\sigma_T}}\ (\Delta\sigma_T - \Delta'\sigma) \qquad (2')$$

and

$$\sqrt{\frac{\Delta'\sigma}{R_0}} = \sqrt{\frac{2K'}{\Delta\sigma_T}} \quad (2\Delta\sigma_T - \Delta'\sigma) \qquad (3')$$

with $\Delta'\sigma = \sigma - \sigma_D$, $\Delta'\sigma_T = \sigma_T - \sigma_D$, σ_T being the chemical shift of the tetramer.

The fig. 6B shows the plot of $(\frac{\Delta'\sigma}{R_0})^{1/2}$ versus $\Delta'\sigma$; this leads to values of $K' = 2.5 M^{-1}$ and $\Delta\sigma_T = 25$ Hz for an n-mers model. If a tetramer model is used the values of K' is 1.25 M^{-1} and $\Delta\sigma_T$ is 50 Hz. This latter seems too high and favours the first model which can be described by the following equations:

$$R + R \leftrightharpoons R_2 \quad K$$
$$R_2 + R_2 \leftrightharpoons R_4 \quad K'$$
$$\text{----------}$$
$$R_{2n} + R \leftrightharpoons R_{2n+2} \quad K'$$

4. Conclusion

Our results clearly demonstrate that Daunorubicin self-associate in solution. At low concentration of Daunorubicin ($C < 5 \times 10^{-8}$ M) all experimental results are in agreement with a dimerization model. The value of the association constant obtained from CD measurements (570 M^{-1}) and the one obtained from NMR experiments (700 M^{-1}) are in good agreement. At higher concentrations more complex association occurs, which leads to drastic changes of the CD spectrum, with large dependence on the temperature. A possible explanation is the self-association of dimers to give n-mers, with an association constant of about 2 M^{-1}.

One must notice that the CD spectrum obtained at high concentration of Daunorubicin and low temperature is very similar to that observed when Daunorubicin bound to DNA with a high antibiotic to phosphate ratio [5]. In this case an external binding was postulated without intercalation in the helix as for many dyes [5,14,15]. The similitudes of the CD spectra strongly suggest that in this type of binding the antibiotics are stacked along the DNA helix.

Acknowledgement

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