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Dedicated to the memory of
Professor Cristofor I. Simionescu (1920–2007)

FLUORESCENCE STUDY OF THE INTERACTION OF ANTITUMOR DRUG ACTINOMYCIN D WITH DOUBLE STRANDED DNA

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Binding of antitumor drug actinomycin D with double stranded DNA has been investigated by fluorescence spectroscopy. The emission changes accompanying the interaction of the drug with DNA were rationalized in terms of the Scatchard and Schwarz models, in order to calculate binding parameters: binding constant, the number of binding sites and the cooperativity parameter. Scatchard plots indicate three distinct processes: two cooperative processes and a non-cooperative process. The results are discussed in comparison with those obtained by absorption spectroscopy.

INTRODUCTION

Actinomycin D is an exhaustively studied antitumor drug that contains a planar 2-aminophenoxazin-3-one chromophore and two cyclic pentapeptide lactones (Fig. 1). This drug has been used clinically for the treatment of highly malignant tumors, such as Wilms' tumor[1] and gestational choriocarcinoma,[2] and in combination with other antitumor agents to treat high-risk tumors.[3,4] Actinomycin D exerts its biological function via inhibition of RNA polymerase and transcription by the intercalation of the planar chromophore, preferably at the GpC sequence, with the two pentapeptide rings resting on the minor groove. A model of double stranded (ds) DNA-actinomycin D complex has been generally accepted in which the phenoxazone ring is intercalated between the GC and CG base pairs, forming strong hydrogen bonds in the minor groove between the guanine 2-amino groups and the carbonyl oxygen atoms of the L-threonine residues of the cyclic peptides.[5,6] Additional stabilizations are derived from hydrophobic interactions between groups on the peptides and sugar residues, and from other specific weaker hydrogen bonds.[7,8] Also, actinomycin D may bind strongly via some non-classic modes, such as to single stranded (ss) DNA[9-12] and to some DNA sequences containing no GpC site.[13-15]

The binding of actinomycin D to dsDNA and ssDNA was discussed previously in the frame of MM^+ molecular mechanics and AM1 semiempirical method. Also, these complexes were analyzed using HBexplore program,[16] based on geometrical criteria and SHB_interactions program,[17] based on quantum-chemical criteria (Mulliken overlap populations). The results outline the contribution of specific hydrogen bonding as well as C-H···O(N) and other atom-atom intermolecular interactions to the stabilization of the actinomycin D-DNA complexes.[18,19]

In previous absorption experiments, the Scatchard plots for the binding of actinomycin D to dsDNA and ssDNA indicated three different processes: I – a cooperative process at low P/D (the ratio between the polymer and drug concentration) values, assigned to the external binding of actinomycin D to nucleic acids; II – a non-cooperative process coresponding to the intercalation of the phenoxazone chromophore of the drug between the base pairs of the nucleic acids; III – a cooperative process at high DNA concentrations (high P/D ratios), tentatively assigned to the interaction of the actinomycin D-DNA intercalation complexes with another ssDNA or dsDNA sequence.[12,18,19]

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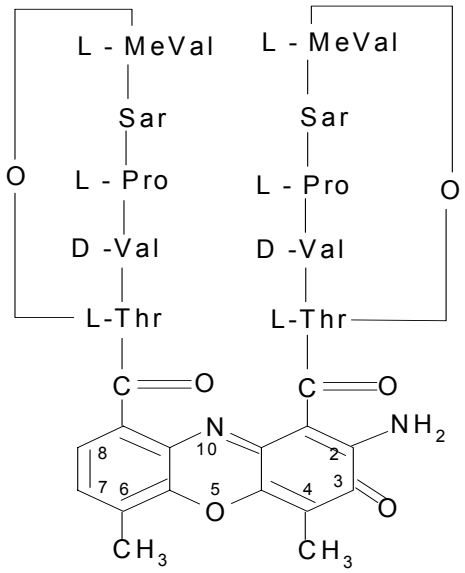


Fig. 1 – The chemical structure of actinomycin D.

The objective of the present work is to investigate the binding of actinomycin D to dsDNA by fluorescence titration experiments and to determine the binding parameters, the binding constant, the size of binding site, the cooperativity parameter.

MATERIALS AND METHODS

Actinomycin D and calf thymus DNA were purchased from Sigma and used without further purification. All solutions were prepared in sodium phosphate buffer (pH 7.2). Concentrations of the solutions were determined spectrophotometrically by using the molar absorption coefficients of ε_{260} = 6600 $M^{-1}cm^{-1}$ (per nucleotide) for calf thymus DNA and ε_{440} = 24400 $M^{-1}cm^{-1}$ for actinomycin D.[21] Fluorescence emission spectra were measured on a FP-6300 spectrofluorometer (λ_{ex} = 380 nm). Spectral titrations were carried out at room temperature by starting with an actinomycin D solution and a progressive addition of small aliquots of DNA solution. Fluorescence measurements were made after a period of time sufficient to ensure attainment of equilibrium. Non linear fitting was performed using Table Curve 3D software and a procedure previously described,[20] based on Schwarz general treatment.[22]

RESULTS AND DISCUSSION

The fluorescence emission spectra of actinomycin D alone and in the presence of DNA at different polymer to drug ratios (P/D) are presented in Fig. 2. In the presence of DNA, the fluorescence emission of actinomycin D is quenched, with no shift in the emission maximum (λ_{em} = 475 nm).

The Stern-Volmer plot (Fig. 3) shows a linear portion up to the DNA concentration of $4.0x10^{-5}$ M, followed by a positive curvature which is usually considered as indicative for a static quenching due to the association process.

The changes in the fluorescence intensity of actinomycin D upon addition of DNA were used to generate binding curves and to determine the binding parameters. If the drug emission is quenched upon DNA binding, the concentrations of free and bound drug may be calculated from the equations:

$$C_f = \frac{C_T(F/F_0 - P)}{1-P}; \qquad C_b = C_T - C_f \qquad (1)$$

where C_T is the known total drug concentration, F_0 and F are the fluorescence intensity of actinomycin D in the absence and in the presence of DNA, and P is the ratio of the fluorescence of the completely bound drug to the free drug, $P = F_b/F_0$. The value of P was determined by measurement of the fluorescence emission of actinomycin D in the absence and in the presence of a large excess of DNA. Equations (1) are based on the assumption that the binding process is two-state, and that the observed spectral response is a linear combination of only two spectral species, corresponding to the free and bound drug.[23]

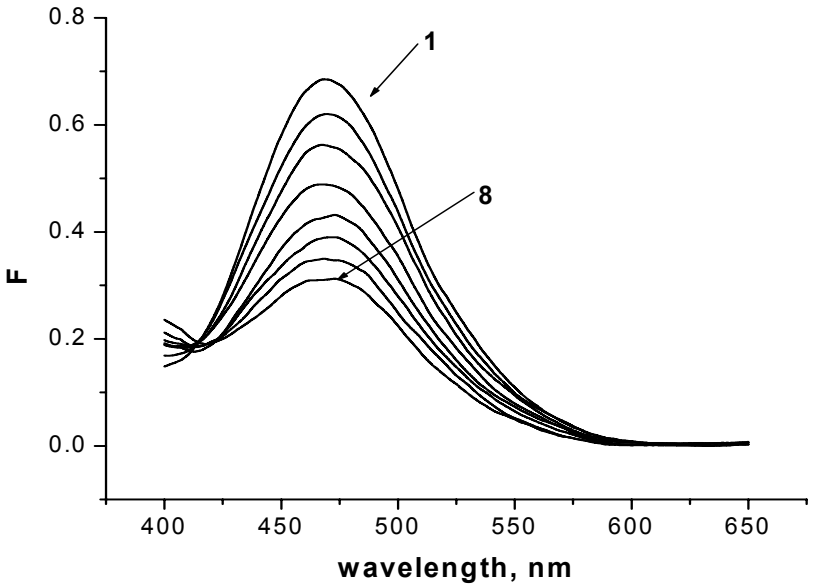


Fig. 2 – The fluorescence spectra of actinomycin D in the presence of double-stranded DNA at different P/D ratios: 0; 4.65; 9.2; 13.7; 18; 22.4; 26.6; 30.7 (curves 1 – 8).

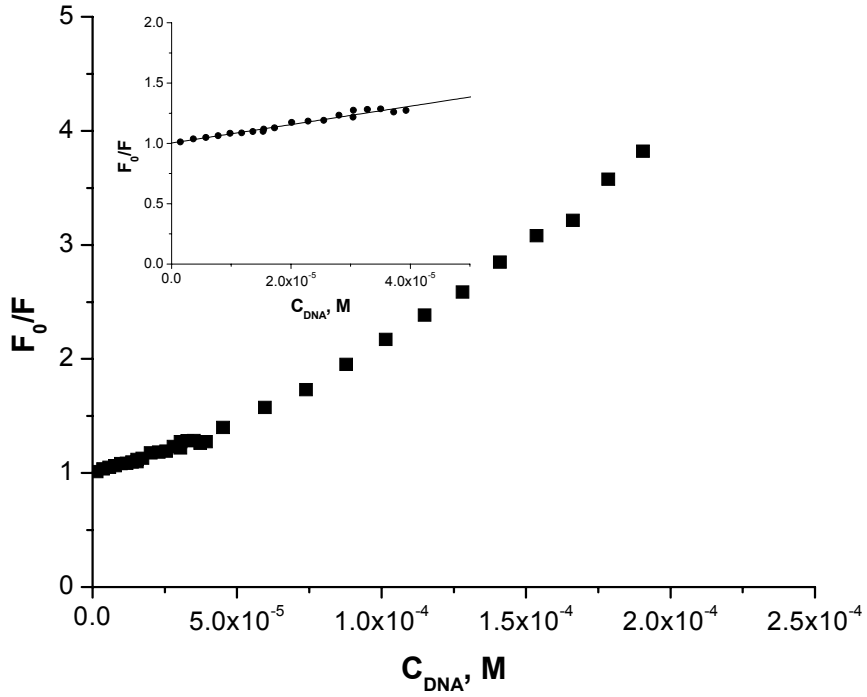
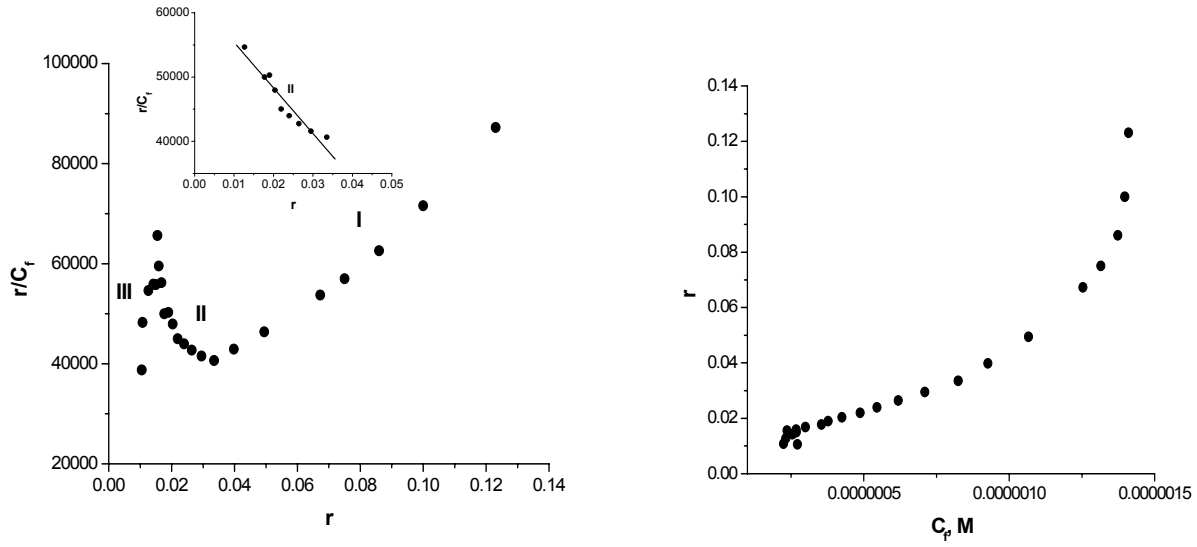


Fig. 3 – Stern-Volmer plot for the actinomycin D – DNA system; inset – the extended plot for the DNA concentration up to $4.0x10^{-5}$ M.

The equilibrium binding isotherms for the interaction of actinomycin D with calf thymus DNA are usually presented in the form of Scatchard plot (Fig. 4) using equation:

$$r/C_f=(n-r)K \quad (2)$$

where r is the molar ratio of bound actinomycin D to the total DNA concentration, n is the number of binding sites per nucleotide and K is the binding constant.[24]



(a) (b)

Fig. 4 – (a) Scatchard plot for the equilibrium binding of actinomycin D to DNA; inset – linear fit of process II according to equation (2); (b) Plot of r vs. the free actinomycin D concentration.

The plots in Fig. 4 evidence three different processes, similar with the previous experimental absorption data: I) at high r values (r: 0.04 – 0.12; P/D = 0 – 12), a positive slope is observed, indicating a cooperative binding; II) at values of r: 0.04 – 0.01 (P/D: 12 – 70), a negative slope is observed, consistent with the neighbour exclusion model for drug binding[25]; III) at low r values (P/D>70) a few points with a positive slope appear, indicating a third cooperative process. This process was better evidenced and characterized by absorption spectroscopy.[20]

In a first step, a separate analysis of processes I and II was performed.

For process II, the binding parameters were straightforward evaluated using equation (2) (Fig. 4 – inset) and the results are presented in Table 1.

For process I observed at high r values (P/D<12), the positive slope of the Scatchard plot indicates a cooperative interaction of actinomycin D with DNA. An estimation of the binding parameters for this process was performed using the model given by Schwarz,[26-29] based on the linear polymeric chain of equivalent binding sites with nearest-neighbour cooperativity. This model assumes two types of intrinsic processes: a) nucleation – the binding of an isolated ligand, characterized by a binding constant $K^{\#}$; b) aggregation – the binding of the ligand in the immediate neighbourhood of one that is already bound, described by a binding constant $K = qK^{\#}$.

The ratio $q=K/K^{\#}$ defines the degree of cooperativity.

In this model, the cooperative binding constant K is given by equation (3):

$$K = (\gamma_D^0 C_T)^{-1} + 2K_d \quad (3)$$

where K_d is the dimerization constant, previously determined ($K_d = 3650\ M^{-1}$)[20] and $\gamma_D = (F - F_b)/(F_0 - F_b)$ is the fraction of free monomeric drug. The equation (3) is valid if the binding of the drug to DNA is stronger than the dimerization tendency of the drug, $K>>K_d$. Taking into account the dimerization process, the total fraction of free drug (γ_D^*) may be calculated as:

$$\gamma_D^* = \gamma_D(1+2K_d C_T \gamma_D) \quad (4)$$

The dependence of the total fraction of free drug (γ_D^*) on P/D ratio is described by the equation (5), where θ is the fraction of occupied binding sites:

$$\gamma_D^* = 1-\theta\frac{1}{n}\frac{P}{D} \quad (5)$$

A plot of γ_D^* as a function of P/D at constant drug concentration allows the determination of the binding parameters, K and n (Fig. 5). The intersection of the extrapolated initial slope with the abscissa yields the reciprocal number of binding sites. The results are presented in Table 1.

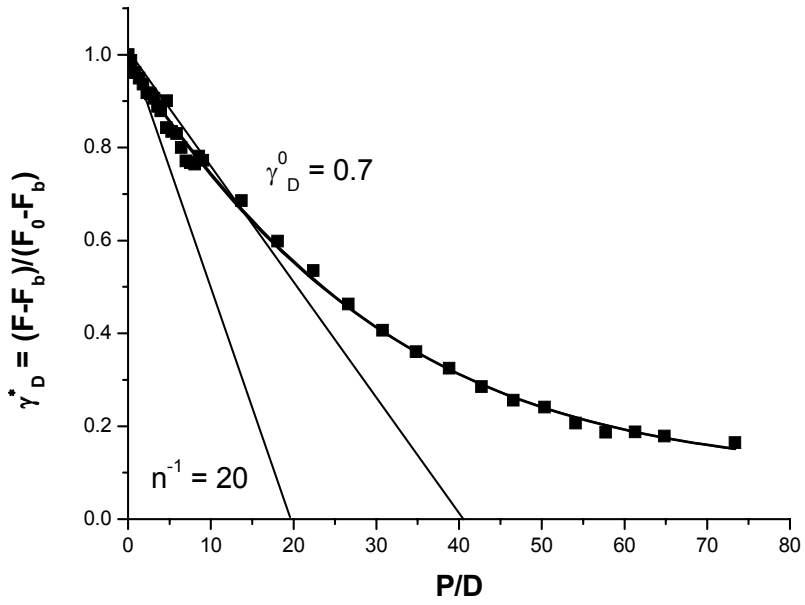


Fig. 5 – Fraction of free actinomycin D vs. polymer to drug ratio at constant concentration of drug, $C_T = 3.2 \times 10^{-6}$ M.

Table 1

Binding parameters for the interaction of actinomycin D with dsDNA, using different methods and models discussed in the text

	Fluorescence spectroscopy			**Absorption spectroscopy**[20]		
Separate analysis	**log K**	**n**	**q**	**log K**	**n**	**q**
process I Ec. (3)	5.50	0.05	-	5.49	0.11	-
process II Ec. (2)	5.88	0.09	-	5.22	0.11	-
General analysis						
process I (cooperative)	5.85	0.23	140	5.06	0.59	451
process II (non-cooperative)	5.96	0.08	0.9	5.20	0.10	0.52

For non-linear fitting of the data corresponding to processes I and II, we used the generalized Schwarz procedure, which takes into account both a non-cooperative and positively cooperative binding.[22] The general relationship depends on the binding constant (K), the cooperativity parameter (q) and the number of binding sites (n). The fit is presented in Fig. 6 and the binding parameters are also included in Table 1.

Analysis of the results in Table 1 points out that: a) similar values for the binding constant for the process I (cooperative) were obtained by separate analysis of both emission and absorption experimental results; b) the binding constants for process I (cooperative) and II (non-cooperative) obtained by general analysis and the binding constant for the process II obtained by separate analysis are higher in the case of emission spectroscopy than in absorption spectroscopy; c) somewhat greater differences are observed between the two methods regarding the binding site size (n) and cooperativity parameter (q). However, the differences between the values of cooperativity parameters are in the range of experimental literature data.[27] The differences between the results obtained by emission and absorption spectroscopy could be due to the different concentration range used in both experimental techniques and, on the other hand, to the complexity of the binding process. In absorption spectroscopy the concentrations of the drug are one order of magnitude higher than in emission spectroscopy, and in this case the self-association of the drug is more important and can affect the binding process.

These spectral (fluorescence and absorption) studies are valuable for a better understanding of the specific mode of actinomycin D-DNA interaction, which should allow a deeper insight into the therapeutic efficiency of actinomycin D and design of new targeted drugs.

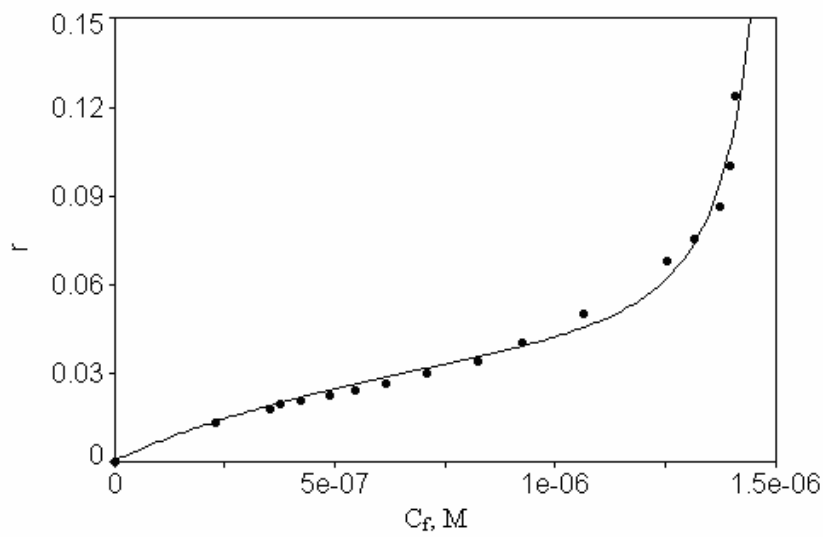


Fig. 6 – Non-linear fitting of the data corresponding to processes I (cooperative) and II (non-cooperative) using Schwarz theory.

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