THE BINDING OF VIOLAMYCIN (V B1) TO SINGLE STRANDED NUCLEIC ACIDS

Tatiana Oncescu* **and Simona Radulescu**

abstract: The binding of violamycin BI to single stranded nucleic acids is a cooperative process leading to the formation of the stacked dye molecules attached at the polyanion surface. We applied Schwarz treatment to determine the binding parameters: the equilibrium constant of the stacked complex formation K_{st} and of the nucleation process K_{nuc} respectively, g the number of binding sites per monomeric unit of the polyanion and q the cooperativity coefficient. The results obtained permitted us to suggest a binding mechanism involving interactions which are discussed in details.

Introduction

The anthracyclin antibiotics are important agents in the treatment of malignant diseases [1, 2]. Their effectiveness is due to their ability to interact with DNA [3] resulting in the inhibition of DNA replication and transcription [4]. Biochemical investigations have indicated that violamycin BI (V BI) inhibits DNA dependent RNA synthesis and the action of repair enzymes in bacteries [5, 6]. In addition V BI inactivates influenza virus [7] and herpes virus [8].

Various papers [9, 10, 11, 12] showed that V BI binds in a complex manner to DNA by specific (intercalation between base pairs) and nonspecific (stacking on the biopolymer surface) interactions. In order to investigate separately these interactions we studied in this paper the binding of V BI to the single stranded nucleic acids: poly (A), poly (G), poly (dT) and poly (dGC), poly (C) being analysed in a previous paper [13]. V BI forms only the stacked complex on the surface of these polyacids. So, we prevent the interference of the stacked complex formation with the intercalated one.

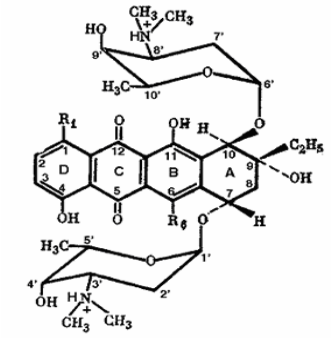
Material and Methods

Violamycin BI was offered by Dr. Strauss D.G who isolated it for the first time from *Streptomyces violaceus* [14].

* Department of Physical Chemistry, Faculty of Chemistry, University of Bucharest, Bd. Regina Elisabeta 4-12, 030018 Bucharest, Romania

Analele Universităţii din Bucureşti – Chimie, Anul XIV (serie nouă), vol. I-II, pg. 321-331

Violamycin BI is an equimolecular mixture of three isomers as follows:



	R_1	R_6
VBI/1	H	OH
VBI/2	OH	OH
VBI/3	OH	H

V BI solutions were freshly prepared in a phosphate-EDTA buffer pH 7 and kept in the dark before measurements to avoid photobleaching. Its concentration was determined by measuring the absorption at λ = 500 nm using ε_{500} = 10250 $M^{-1}cm^{-1}$ [15].

The synthetic nucleic acids: poly (G), poly (A), poly (dT) and poly (dGC) were purchased as potassium salts from SIGMA. Their solutions were prepared in the same buffer and the concentrations determined by measuring the absorbances at λ = 257 nm using their characteristic molar absorption coefficients as follows: 9500, 10500, 8700 and 6600 $M^{-1}cm^{-1}$ respectively [16]. The absorption spectra were measured at an Unicam UV/VIS α Helios spectrophotometer and the fluorescence ones at an Aminco-Bowman spectrofluorimeter. For the determination of the binding parameters we carried out the direct as well as the inverse titration into the spectral cells. The addition of the polyacid into V BI solution or of V BI into polyacid in the measure cell was accompanied each time by the addition of an equal volume of buffer in the reference cell to prevent the differences between the two cells due to the dilution of the matrix.

Results and Discussions

The surface complex

We exemplify in Fig. 1 our spectrophotometric measurements resulted from V BI titration with poly (A).

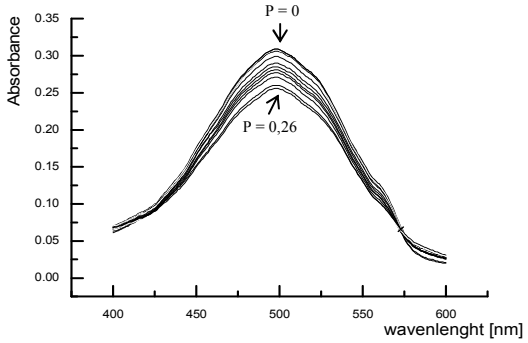


Fig. 1. *The evolution of the absorption spectra during the titration of V BI with poly (A) at small values of p.*

One observed a hypochromic effect with a slight shift to the longer wavelengths and an isosbestic point at λ = 572 nm. This behavior is similar to V BI – poly (C) system [13] assigned to the stacked complex formation of V BI on the polyacid surface. The interactions are of electrostatic nature because V BI monomer at pH 7 carries two positive charges on the nitrogen of the glucosyl residues, attracted by the negative PO_4 groups of the biopolymer. This binding involves processes where dye molecules interact in the stack in a cooperative manner. Therefore we adopted the basic model of Schwarz [17] with just one type of equivalent binding site what restricts cooperative interactions to those with the nearest neighbors. Also the other polyacids that we studied showed a similar behavior as poly (A).

From fluorescence spectra, using λ = 500 nm for violamycin excitation one observed the quenching of the fluorescence intensity during the titration in the same range of p ($c_{poly}/c_{V\,BI}$) as in absorption, what confirmed the formation of the stacked complex.

In Fig. 2 one compares the evolution of V BI-poly (A) system during the titration, as γ vs p, γ being the fraction of the free monomeric V BI in solution, resulted from A/A_0 and I/I_0 respectively.

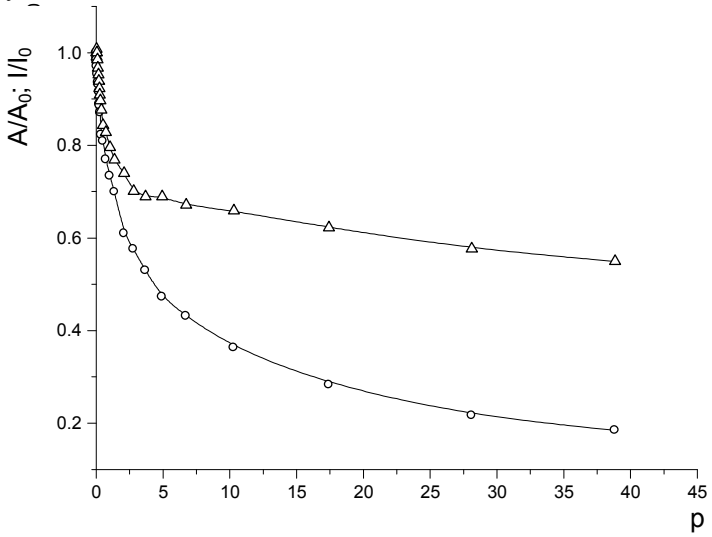


Fig. 2. *The evolution of γ during the titration in absorption (△) and fluorescence emission (○) of V BI.*

One remarks that γ decreases much more in emission that in absorption due to the fact that the stacked complex absorbs in the same spectral range as the free violamycin but with an lower ε. In exchange only the free monomeric V BI emits fluorescence, not at all its stacked complex. Therefore we preferred to use the fluorescence measurements to the evaluation of our experimental data for the accuracy of the results.

In our computation we took also into account for the possible dimers of V BI in solution, by using equation (1):

$$\gamma^{*} = \gamma(1 + 2K_d c^0_{VBI}\gamma) \tag{1}$$

γ^* being the total fraction of free ligand (monomers and possible dimers) and K_d the equilibrium constant for V BI dimerization at pH 7 and the natural ionic strength of the buffer 0.02 M [18]. In Fig. 3 is shown the evolution of the titration in the range 0 to 18 of p.

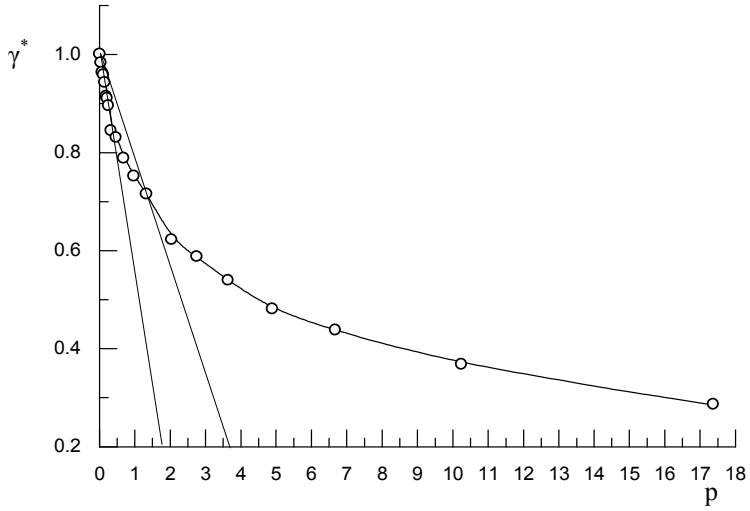


Fig. 3. *γ^* vs p at the titration of V BI with poly (A); c^0_{VBI} = 3 x 10^{-5} M*

The fraction of V BI bound in stack is given by:

$$\theta gp = 1 - \gamma^* \tag{2}$$

in which θ is the fraction of binding sites occupied by V BI (saturation degree) and g is the number of binding sites per monomeric unit of the polymer.

At the beginning of the titration γ^* is proportional to p as Fig. 3 shows. If one prolonges this linear part up to the abscissa, the intercept gives pg = 1 and hence one obtained the binding parameters g = 0.56. At the first sight it seems to be a plausible value because V BI has two positive charges and the monomeric segment of the nucleic acid containing a PO_4 group offers only one negative charge. It should be mentioned that the concentration of the monomeric segments defines the nucleic acid concentration c_p. Thus with g binding sites per segment, the total concentration of binding sites is gc_p, in molar phosphate.

Once g is known a straight line of slope –g/2 can be drawn as shown Fig. 3, which intercept the binding curve at $\gamma^* = 0.68$. The theory [17] shows that for this point γL=1, L being the binding strength of V BI in the stacked complex. It depends on the initial concentration of V BI as follows:

$$L = K_{st} c^0_{VBI} \tag{3}$$

in which the binding equilibrium constant K_{st} of V BI in the stacked complex can be immediately calculated and found 4.9 x 10^4 M^{-1}, L being 1.47.

The value of K_{st} was deduced also by other computation method using the same experimental data, namely by plotting the equation (4) as in Fig. 4:

$$\frac{(1-2\theta)\sqrt{c_{VBI}^{free}}}{\sqrt{\theta(1-\theta)}} = \sqrt{\frac{q}{K_{st}}} - \sqrt{qK_{st}} \times c_{VBI}^{free} \tag{4}$$

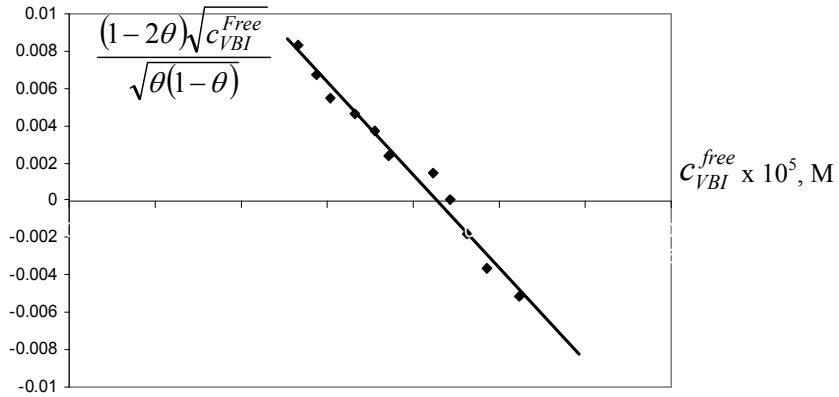


Fig. 4. *Determination of the binding parameters according to the equation (4)*

The straight line has two intercepts: with abscissa $1/K_{st}$ and with the ordinate $\sqrt{q/K_{st}}$. One obtained in this way K_{st}=4.7 x 10^4 M^{-1} in good agreement with the above one. At the same time one computed also q = 21.2.

If one compares this K_{st} value with that obtained at the binding of V BI to poly (C), 3.25 x 10^4 M^{-1}[13] one observes that the last one is lower. At the first sight it is surprising because in both cases these values refer to the binding of V BI in stacked complex on the single stranded acids in which the distances between two PO_4 is practically the same. Hence it was expected very near values of K_{st}. Therefore we determined this constant also for the other single stranded acids and found the values summarized in Table 1.

Table 1. The binding parameters of V BI to single stranded nucleic acids in phosphate-EDTA buffer, pH 7.

Polyacids	g	$K_{st}/10^4$, M^{-1}	ΔG_{st}, kJ	q	$K_{nuc}/10^4$, M^{-1}
poly (G)	0.50	8.72	-28.19	6.4	1.36
poly (C)	0.43	3.25	-25.74	13.0	0.22
poly (A)	0.53	4.80	-26.50	21.2	0.23
poly (dT)	0.62	3.82	-26.14	20.0	0.19
poly (dGC)	0.50	12.00	-28.98	9.0	1.33

In Table 1 appears also the binding parameters for a bibasic single stranded acid, poly (dGC) where K_{st} presents a cumulative value from $K_{st}^{p(G)}$ and $K_{st}^{p(C)}$, namely 12 x $10^4 M^{-1}$.

One observes indeed, very different values of K_{st}. This suggests us that besides the stacked complex formation due to the electrostatic forces, probably interactions with the different bases of the polyacids take also place.

From Table 1 results the following decrease order of K_{st} values:

$$K_{st}^{p(G)} \rangle K_{st}^{p(A)} \rangle K_{st}^{p(dT)} \rangle K_{st}^{p(C)} \tag{5}$$

With the values of K_{st} one calculated ΔG_{st}= -RTlnK_{st} and found the same serie as (5).

Dourlent and Hélène [19] investigated the binding of proflavin to single stranded poly(A) and found that not only the outside binding as aggregate on polymer takes place but also a dye-base staking occurs. The first binding was cooperative whereas the second one anticooperative. Later Schwarz and von Tscharner [20] studied the binding of acridine orange to poly (A). They demonstrated that AO bound to poly (A) by nucleation can intercalate by twisting around the backbone and stacking with two adiacent bases. So, there is competition at the same binding sites of the polyacid for intercalation and cooperative binding. One can conclude that our assumption that interactions with the bases have to take place has sufficient support.

On the other hand one notices in Table 1 that g is of about 0.5 in all cases. We have to keep in mind that the distance between two positively-charged centers in V BI is about 12 Å [13, 21] whereas the distance between the two consecutive negative centers on the phosphate backbone of the polyacid, only of about 6 Å. A structure in which V BI molecules form a staggered stack with their antracycline moieties superposed in a staircase-like pattern, leading to two helical arrangements of positively-charged glucosylic residue on the outside, stabilized by the negative charges of the polyacid located between the two helices, was already suggested for V BI-poly (C) system [13]. Such an arrangement seems to be plausible also in the case of the other single stranded acids from Table 1.

This arrangement of course is achieved with entropy loss what shows that the entropic factor may be predominant in the values of ΔG_{st}.

The cooperativity parameter q permitted the calculation of the nucleation equilibrium constant K_{nuc} from the equation (6):

$$K_{nuc} = \frac{K_{st}}{q} \tag{6}$$

The last column of Table I contents the values of K_{nuc}.

An interesting feature presents the behavior of V BI binding to single stranded nucleic acids at very high values of p obtained by an inverse titration. We exemplify it for poly (G) with V BI in Fig. 5.

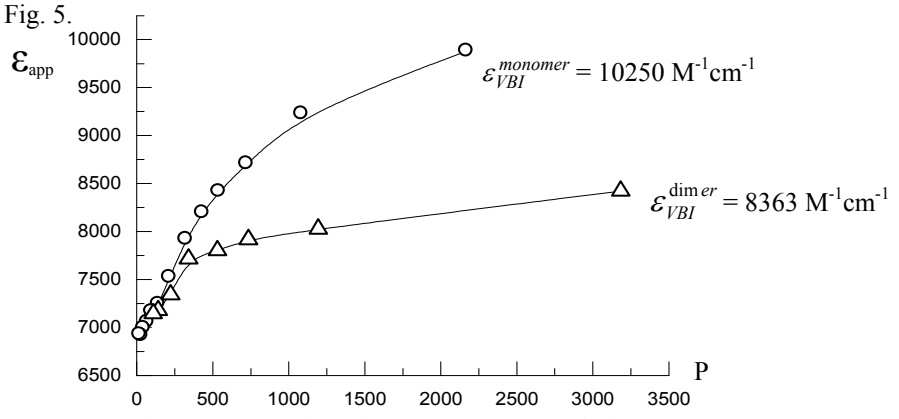


Fig. 5. *The curve ε_{app} vs p obtained by an inverse titration of poly (G) with V BI.*

At very high value of p (>2000) the molar absorption coefficient ε_{app} of V BI-poly (G) system dissolved in water, tends to reach the value 10250 $M^{-1}cm^{-1}$ characteristic to the absorbtion coefficient of the free monomeric violamycin in solution. The same system in phosphate-EDTA buffer don't pass over 8363 $M^{-1}cm^{-1}$, characteristic to the free dimers of V BI. This behavior was put into evidence firstly for V BI - poly (C) system [13, 21] and now confirmed by the other single stranded nucleic acids. One can conclude that in water at high value of p one forms only the nucleation complex that is the monomeric V BI bound isolated on the biopolymer. The absorption spectrum is practically similar to that of the monomeric V BI in water because the electronic structure of the chromophore underwent relatively little change after the attachment of monomer had taken place [12]. Contrary in the phosphate-EDTA buffer, pH 7 and a natural ionic strength of 0.02 M, it forms isolated dimers on the chain in the same range of p. This complex is very stable because even high amounts of polymer don't break it. This high stability of the dimer complex may be explained by the fact that also the free dimer in solution (in the absence of polymer) shows an increasing of the dimerization constant K_d with the increasing of the ionic strength [18, 21].

The binding mechanism

We have to mention that all the nucleic acids presented a modification of the absorption spectra at an higher p than that characteristic to the stacked complex.

Fig. 6 shows this behavior exemplified by V BI – poly (dT) system. The absorption spectrum change suggests again the interaction with the bases, at moderate values of p.

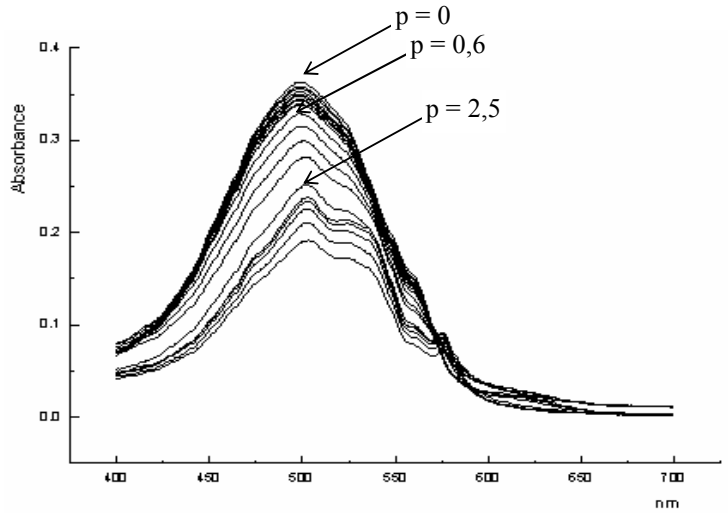


Fig. 6. *The absorbtion spectra of V BI - poly (dT) system at various values of p.*

Indeed, the spectra obtained at small values of p (0 – 0.6) correspond to the formation of the staked complex. These spectra are similar to that of free violamycin but the absorbance decrease with the increasing of poly (dT) concentration because V BI is bound in the stacked complex. As long as poly (dT) concentration increases (and p, too) V BI molecules

are teared off from the aggregates and bound as isolated monomer to the new chains. In this location, as Schwarz [20] propose, V BI molecule twists easily around the backbone and intercalates between two adjacent bases. When the concentration of this species is sufficiently high (p > 2.5) it becames spectral visible by the splitting and shifting of the absorption maximum. This aspect is obtained also when V BI binds to DNA and to other double stranded nucleic acids where the intercalation between the base pairs is a known interaction. With the increasing of p increases also the number of the isolated V BI on the chains and appear the posibility to form isolated bound dimers even on the chains with intercalated V BI. To analyze the behavior of these systems we applied also Scatchard treatment although this was developed for double stranded acids, especially for DNA helix to determined the intercalation equilibrium constant K_{int} according to the equation (7):

$$\frac{r}{c_{free}} = K_{\text{int}}(B - r) \text{ that is } \frac{r}{c_{free}} = BK_{\text{int}} - K_{\text{int}} r \tag{7}$$

r is the ratio of bound dye to base pairs c_{bound} / c_{poly}, B the apparent number of binding sites per base pair, c_{free} the concentration of free dye in solution and c_{bound} the concentration of bound dye. By plotting equation (7) one obtained K_{int} from the slope and B from the ordinate.

Such a plot revealed us as Fig. 7 shows exactely three linear segments characteristic to the three range of p discussed before in connection to the spectral characteristics.

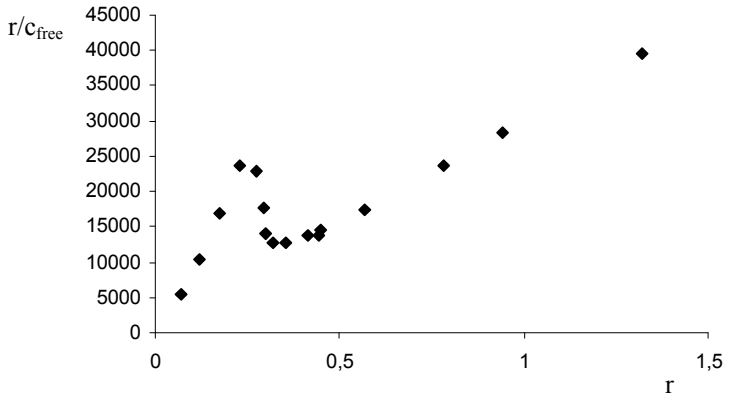


Fig. 7. *Scatchard plot of the V BI - poly (dT) system.*

If one follows the plot from the higher to lower values of r one distinguishes: 1) the cooperative binding (positive slope) of V BI to poly (dT) in stacks; 2) the anticooperative binding (slope negative as eq. (7) requires) which characterises the intercalation of V BI between two adjacent thymines and 3) the formation of dimers bound to poly(dT), again cooperative, which interfer with the intercalated V BI.

The evaluation of these three regions gives the following data: K_{st} = 3.82 x $10^4 M^{-1}$ (Schwarz method) and K_{st} = 2.9 x $10^4 M^{-1}$ (Scatchard method). In addition one calculated

also the stability constant of the intercalated complex K_{int} = 1.4 x $10^5 M^{-1}$ and of the isolated dimeric V BI on polyacid K_D =1.2 x $10^5 M^{-1}$.

One observes that the stacked complex has a value some smaller than that calculated by Schwarz method because Scatchard don't take into account for cooperativity.

One remarks that the intercalated complex is five times stable than the stacked complex because besides the atractive electrostatic forces contributes also the interaction with the bases to its stability.The same bahavior was evidenced also by the other nucleic acids.

Löber, Kittler [22, 23] studied the fluorescence quenching of various dyes by DNA and assigned it to the electron transfer from DNA bases to the dyes in their excited singlet state. In addition they studied also the fluorescence quenching of two antracycline antibiotic (daunomicin and violamycin BI) with poly (A). Their reduction potentials 1.35 V and 1.25 V respectively indicated that the electron transfer from adenine (the oxidation potential 0.85 V) is possible.

We thought that if V BI intercalates between two adjacent bases, this location approaches the electron donor-acceptor couple and favorizes the charge transfer. In consequence we analyzed the fluorescence quenching of V BI from this point of view. With other words if the quenching of fluorescence emission is due to the electron transfer, Stern Volmer type equation has to be fullfield:

$$\frac{I_0}{I} = 1 + k_{SV}[Q] \tag{8}$$

I_0 and I are the fluorescence intensity emitted by V BI in the absence and presence of the quencher Q; k_{SV} is Stern Volmer constant resulted from the slope of this equation. We exemplify by V BI - poly (dT) system in Fig. 8.

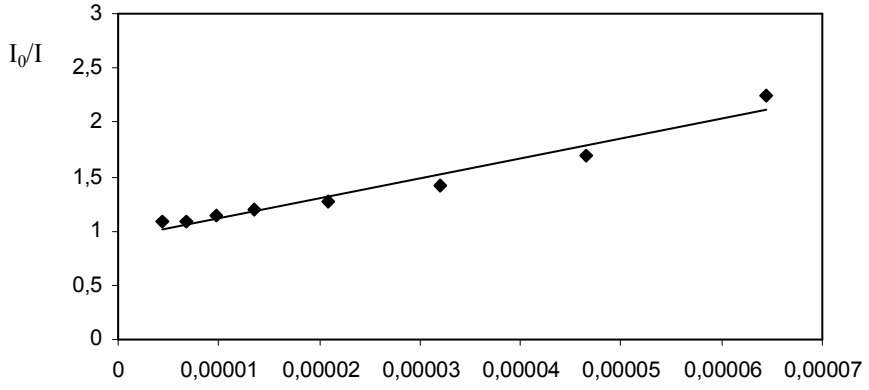


Fig.8. *Stern Volmer plotting for V BI - poly (dT) system.*

But $k_{SV} = k_q \tau_{V\ BI}$ in which k_q is the rate constant of fluorescence quenching that is of the electron transfer process and $\tau_{VB\ I}$ is the life time of V BI in its excited singlet state. This is equivalent to the product $\tau_0 \Phi_{em}$ in which τ_0 is the radiative life time of V BI in its excited singlet state and Φ_{em} the emission quantum yield of V BI known from literature, 0.23 [12].

To determine τ_0 we used the absorption spectrum of V BI by changing the coordinates A vs λ with ε vs $\tilde{\nu}$ and evaluating the area of this last spectrum because this is present as an integral in the equation 9:

$$\tau_0 = \frac{3{,}5 \times 10^8}{\tilde{\nu}_m^2 \int \varepsilon d\tilde{\nu}} \tag{9}$$

One obtained in this way τ_0 = 1.8 x 10^{-8}s and then immediately $\tau_{V\ BI}$ = 4.1 x 10^{-9}s. Once $\tau_{V\ BI}$ known one can calculate k_q. In Table 2 we present the results obtained for all nucleic acids.

Table 2. The quenching rate constants k_q

Nucleic acids	K_{SV} 10^4, M^{-1}	K_q 10^{12}, $M^{-1}s^{-1}$	$U_{oxid.pot}$, V
poly (G)	2.10	5.1	+0.85
poly (A)	0.86	2.1	+0.95
poly (dT)	1.20	2.9	+1.05
poly (dGC)	8.80	21.9	-

Firstly one observes that the rate constants k_q have a high order of magnitude 10^{12}, characteristic to the very fast processes. This is in agreement with our assigning of the quenching process to the electron transfer one.

From the Table 2 results the following series for k_q of the single stranded monobasic acids:

$$k_q^{pG} \rangle k_q^{pdT} \rangle k_q^{pA} \tag{10}$$

In the last column of the table figure the oxidation potentials of the bases [23]. It was concludent that this series varies according to the oxidation potentials, but as one can see there is an inversion between poly (dT) and poly (A) values.

On the other hand the literature [24] offers also the oxidation potentials of these bases which support the series of k_q established by us.

In the last line of the table is given the value k_q for the bibasic single stranded acid poly (dGC) which has the highest value. Unfortunately we didn't find the oxidation potential of this base to can compare it with the other.

Conclusions

The study of violamycin BI binding to the single stranded nucleic acids revealed:

- the formation, due to the electrostatic forces, of the stacked complex on the polyacid surface in a cooperative manner at very low value of p (c_p/c_{VBI});
- the anticooperative intercalation of V BI between two adjacent bases with a possible electron transfer from the base to violamycin, at moderate value of p;
- the formation of the isolated monomers V BI on the polyacid chain (nucleation complex) at very high value of p in distillated water and of the isolated dimeric V BI in phosphate-EDTA buffer, a very stable complex.

REFERENCES

1. Arcamone, F. (1987) *Topics Antibiotic Chemistry* **2**, 49-54.
2. Strauss, D. G. (1978) *Z. Allg. Mikrobiol* **17**, 459-464.
3. Zunino, F., Gambeta, A. , DiMarco, A. and Zaccane, A. (1972) *Biochim. Biophys. Acta* **277**, 489-498.
4. Di Marco, A., Arcamone, F. and Zunini, F. (1975) *Antibiotics* **3**, 101-128.
5. Jacob, H.E., Fleck, W. and Strauss, D. G. (1977) *Z. Allg. Mikrobiol* **17**, 569-573.
6. Störl, K., Flek, W. and Strauss, D. G. *Z. Allg. Mikrobiol* **17**, 459-464.
7. Tasca, O., Burducea, O., Popa, L., Repanovici, R. and Löber, G. (1979) *Rev. Roum. Med-Virol* **30**, 231-232.
8. Rafschlöger, J. and Waschke, S. R. (1979) *Int. Symp. On Antibiotics. Weimar Abstract* 1-17.
9. Löber, G., Kleinwächter, V., Balcarova, Z., Klarner, R. and Fleck, W. (1978) *Studia Biophys* **77**, 205-206.
10. Löber, G., Klarner, R., Smekal, E., Balcarova, Z., Kleinwächter, V., Raim, T., Popa, L. M., Hanschmann, H. and Koudelka, J. (1980) *Studia Biophys* **81**, 103-104.
11. Popa, L. M., Repanovici, R. and Löber, G. (1981) *Studia Biophys* **83**, 113-122.
12. Löber, G., Klarner, A., Smekal, E., Raïm, T., Balcarova, Z., Koudelka, J. and Kleinwächter, V. (1983) *Int. J. Biochem*. **15**, 663-673.
13. Oncescu, T., Stefan, M. and DeMayer, L. (1996) *Biophys. Chem.* **63**, 55-65.
14. Fleck, W., Strauss, D. G., Koch, W., Kraner, P. and Prauser, H. *DDR Patentschrift* **100494,** 1978, Int. Cli. 12d. 9/14.
15. Oncescu, T., Iliescu, I. and DeMayer, L. (1993) *Biophys. Chem.* **47**, 277-
16. Janik, B., **Phisicochemical Characteristic of Oligonucleotides and Polynucleotides** (1974) IFI/Plenum, N.Y.
17. Schwarz, G. (1970) *Eur. J. Biochem*, 442-452.
18. Oncescu, T., Memedula, S. and DeMayer, L. (2000) *Biophys. Chem*.
19. Dourlent, M. and Hélène, C. (1971) *Eur. J. Biochem*., 86-95.
20. Von Tsharner, V. And Schwarz, G. (1979) *Biophys. Struct. Mechanims*, 75-90.
21. Memedula, S. (2003) Doctor Thesis.
22. Löber, G. and Kittler, L. (1978) *Stud. Biophys*. **73**, 25-30.
23. Kittler, L., Löber, G., Gollmick, F. A. (1980) *J. Electroanal. Chem., **116**, 503-511.*
24. Steenken, S. and Jovanovic V.S. (1997) *J. Am. Chem. Soc.,* 119, 617-618.