A Kinetic Study of the Intercalation of Ethidium Bromide into Poly(A)·Poly(U)

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The equilibria and kinetics of the intercalation of ethidium bromide (EB) into poly(A)·poly(U) have been studied in aqueous solution (pH = 7.0, 1 M NaCl, 25 °C) by spectrophotometry, spectrofluorimetry, and stopped-flow techniques. The analysis of the binding isotherms yields the site binding constant $K = 2.6 \times 10^4$ M^{-1} and the site size $n = 2.2$ base pairs, thus revealing the occurrence of intercalation according to the excluded site model. The salt dependence of the binding constant indicates that approximately one Na^+ counterion is displaced from the polymer by the binding of one ethidium ion. The kinetic curves are biexponential both in absorbance and in fluorescence. The results suggest the formation of two bound forms according to the reaction scheme $D + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrows}} DS_I$ and $S + DS_I \underset{k_{-2}}{\overset{k_2}{\leftrightarrows}} DS_{II} + S$, where $k_1 = 5.9 \times 10^4$ M^{-1} s^{-1}, $k_{-1} = 8.7$ s^{-1}, $k_2 = 1.8 \times 10^5$ M^{-1} s^{-1}, and $k_{-2} = 0.70 \times 10^5$ M^{-1} s^{-1}. The value of the equilibrium constant K, derived from kinetics as a combination of rate constants, agrees with that derived from equilibria. The behavior of the investigated RNA/ethidium system is compared with that of the DNAs/ethidium systems.

Introduction

The dynamics of the interaction of ethidium bromide (EB) with the DNA double helix have been extensively studied by a variety of techniques,[1−11] but without fully converging conclusions. It is generally recognized that the main step of the interaction is the intercalation of the planar phenanthridinium ring between the base pairs of the deoxyribonucleic acids, but different hypotheses have been proposed about the details of the process, and about the total number of the steps involved in the mechanism of the process itself. It should be noted, however, that the results from different studies could not be easily compared due to the different experimental conditions used in the investigations.

In contrast, the kinetic aspects of dye binding to RNA, either in single or double strand form, have been far less investigated. Rate measurements of the ethidium binding to transfer RNA[12] and of the proflavine binding to single stranded poly(A)[13] reveal positive cooperativity, whereas the kinetic features of the interactions of proflavine with poly(A)·poly(U)[14] and of Pt−proflavine with double stranded poly(A)[15] indicate that intercalation according to the excluded site model is prevailing.

Ethidium is one of the most commonly employed nucleic acid staining agents and the thermodynamic aspects of its binding to polyribonucleotides have received some attention.[16−19] However, the mechanism of the interaction of this important dye with double-stranded RNA has not yet been investigated. This issue seems to be of particular interest; hence, we have investigated the equilibria and the kinetics of the interaction of EB with poly(A)·poly(U), a synthetic RNA that could constitute a good starting point for further kinetic studies with more complex RNA sequences.

Materials and Methods

Materials. Ethidium bromide of analytical grade from Sigma (St. Louis, USA) was recrystallized from water. Stock solutions were then prepared by dissolving appropriate amounts of the solid in doubly distilled water and kept in the dark at 4 °C. It should be mentioned, however, that solutions of not recrystallized EB behave similarly to solutions prepared with the purified solid. The concentration of EB was measured at $\lambda = 480$ nm by using for the molar extinction coefficient ϵ the value of 5700 M^{-1} cm^{-1} in 0.1 M NaCl.[10] The values of the molar extintion coefficient of ethidium bromide have been measured for NaCl concentrations between 0.1 and 1 M and found to change with the salt concentration according to the empirical equation $\epsilon_{EB} = 5679 + 324$[NaCl].

Poly(A)·poly(U) was purchased from Pharmacia Biotech (Uppsala, Sweden) in the form of lyophilised sodium salt and used without further purification. Poly(A)·poly(U) was also prepared by mixing equimolar solutions of poly(A) and poly(U) from Sigma, both at pH = 7.0 and NaCl 10^{-2} M and allowing the mixture to react for several hours. The spectra of the polymer solutions prepared in this way were found to coincide with the spectra of poly(A)·poly(U) from Pharmacia. Sodium chloride was employed to adjust the ionic strength I of the solutions; sodium cacodylate 2.5×10^{-3} M was used to keep the pH of the solutions at the value of 7.0.

The concentration of the stock solutions of poly(A)·poly(U) from Pharmacia was determined spectrophotometrically by knowing from the analysis certificate that A_{260} units/mg = 15.1 at pH 7.0. The concentrations of the solutions of poly(A) and poly(U) used to prepare the double stranded polymer were measured spectrophotometrically by using $\epsilon = 1.01 \times 10^4$ M^{-1} cm^{-1} at 257 nm, pH = 7.0, and 0.1 M NaCl for Poly(A) and $\epsilon = 8.9 \times 10^3$ M^{-1} cm^{-1} at 260 nm and pH = 7.0 and 0.1 M NaCl for poly(U). The temperature and ionic strength of this work were such that the triple helix (poly(A)·2poly(U)) could not form.[20] All poly(A)·poly(U) concentrations are reported in units of mole of base pairs per dm^3.

Methods. Measurements of pH were made using a Radiometer Copenhagen (Copenhagen, Denmark) PHM-84 pH-meter equipped with a combined glass electrode in which the liquid junction was a 3 M NaCl solution.

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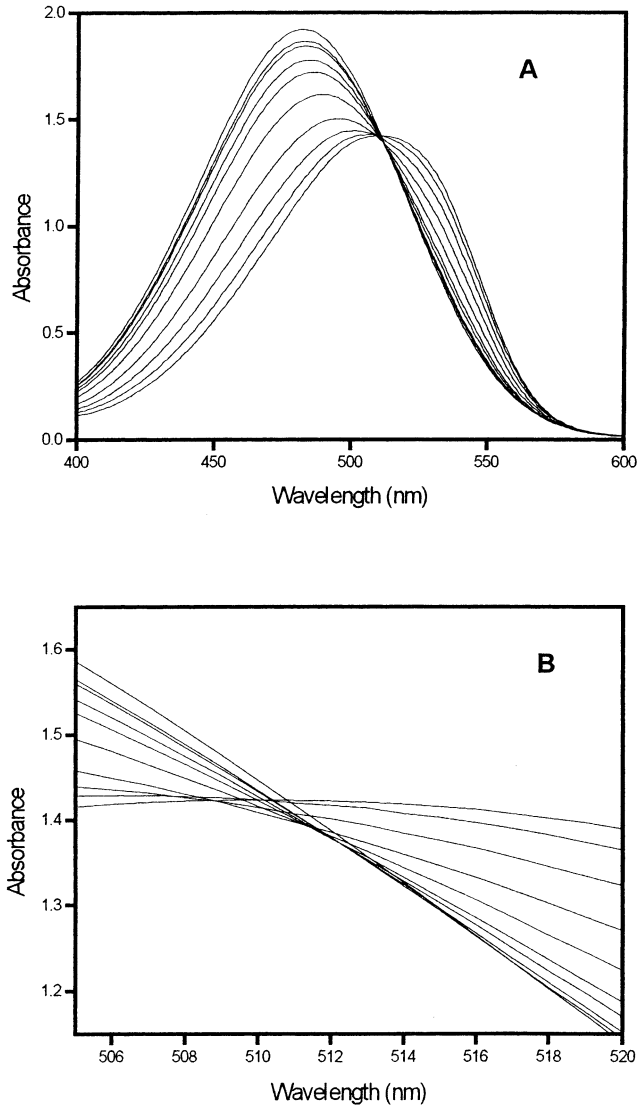

Figure 1. Absorption Spectra of the poly(A) poly(U)/EB system: pH = 7.0, I = 1.0 M (NaCl), T = 25 °C. (A) from top at 480 nm: C_P/C_D = 0.0, 0.27, 0.42, 0.77, 1.10, 1.76, 2.63, 3.51, 4.39, 5.27. (B) Enlargement showing that the isosbestic point, is not well defined.

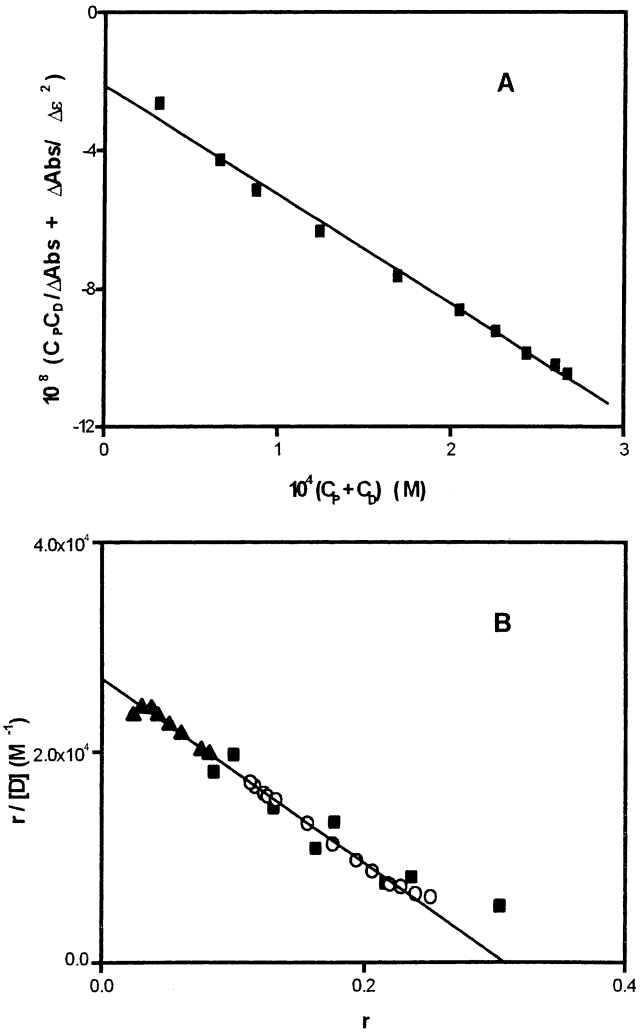

Figure 2. Analysis of the equilibria of the poly(A)·poly(U)/EB system: (A) Titration at constant degree of saturation. r = 0.163, pH = 7.0, I = 1 M (NaCl), T = 25 °C, λ = 480 nm. The reciprocal slope provides the value of $\Delta\epsilon = \epsilon_{\text{bound dye}} - \epsilon_{\text{free dye}}$ whereas the ratio of slope to intercept yields the value of $K(r)$, a binding constant dependent on r. (B) data from absorption (○), fluorescence (▲), and constant r titrations (■), collected in a single Scatchard plot.

Spectrophotometric measurements were made by a Perkin-Elmer & Co. GmbH (Überlingen, Germany) Lambda 17 UV/vis spectrophotometer. The reference cell contained buffer and salt. The linearity of the Lambert−Beer's law for solutions of ethidium bromide was verified between 3×10^{-6} and 3×10^{-4} M. In this range of concentrations the absorption characteristics provided no evidence for dye aggregation. No noticeable adsorption on the surfaces of the spectrophotometer cuvettes was detected. The binding equilibria were first studied in the visible region, where the free nucleic acid does not absorb. When EB interacts with poly(A)·poly(U), its visible absorption spectra shows a pronounced shift to longer wavelengths which indicates a dye−base interaction (Figure 1A). Most of the titrations were carried out by gradual additions of poly(A)·poly(U) to a dye solution, but some titrations were performed by adding EB to a polymer solution: the equilibrium was attained rapidly after each addition and results obtained by the two procedures were identical. Several titrations were also performed at constant values of the binding ratio r (the molar ratio of bound ethidium ion to the total polymer concentration), by adding the dye and the nucleic acid in suitable proportions to the same cuvette containing initially only the buffer.

The dye concentration C_D to be added to a given polymer concentration C_P to obtain a selected and constant value of the [PD]/C_P ratio was evaluated, for each titration point, by using an approximate but reasonable value of $K(r)$. Then, the titration was performed and the measured absorbances were analyzed according to eq 2. Figure 2A shows that, contrary to experiments carried out at variable r values, the points lie now an a straight line. The obtained value of $K(r)$ is used to evaluate r for each point of the titration. It has been found that the r value displays a slight increase as the titration proceeds. However, the maximum variation of r remains within 5% for most of the experiments. Hence, the average of the [PD]/C_P values determined for each titration point was used to express the r value.

Fluorescence titrations were performed on a FP-770 (Japan Spectroscopic Co. Ltd, Tokyo) spectrofluorimeter with the same procedure used for spectrophotometric experiments. The excitation wavelength was λ_{ex} = 510 nm and the signal was measured at λ_{em} = 586 nm where the emission spectrum of the poly(A)·poly(U)/EB system shows its maximum value.

The kinetic measurements were carried out on a stopped-flow apparatus constructed in our laboratory, with detection both in absorbance and in fluorescence. A Hi-Tech SF-61 mixing

unit was coupled to a spectrophotometric line through two optical guides. The radiation produced by a 75 W Hamamatsu L248102 "quiet" lamp or by a 100 W tungsten lamp was passed through a high intensity monochromator and then split in two beams. In the absorption mode the reference beam was sent directly to a 1P28 photomultiplier. The measuring beam was sent through an optical quartz guide to the observation chamber and then, through a second optical guide, to the measuring photomultiplier, also 1P28. In the fluorescence mode the reference photomultiplier was replaced by a constant voltage whereas the measuring sensor was a Hamamatsu avalanche photodiode directly matched to the observation cell and positioned perpendicular to the excitation beam. The reference and measuring voltages were balanced before each shot. The signal revealing the course of the reaction was sent to a Tektronix 2212 digital oscilloscope with a maximum sampling rate of 4000 data points in 200 microseconds and a minimum sampling rate of 4000 data points in 500 s. Finally, the acquired signal was trasferred to a personal computer via a GPIB interface and analyzed by a nonlinear least-squares procedure expressly devised to fit multiexponential functions.[21]

Each time constant used in the analysis of the data is the average of at least six repeated experiments with a maximum spread of 10%.

Results

Equilibria. The absorption spectra, recorded during titration, do not show a well definite isosbestic point (Figure 1B), thus suggesting that the binding process is not simple.

The equilibria were analyzed on the basis of the apparent reaction

$$D + S \leftrightarrows DS_T \qquad (1)$$

where S is the polymer, D the free dye, and DS_T are the total bound forms of the dye.

The binding parameters of the poly(A)·poly(U)/EB system where first measured by spectrophotometric titrations where the extent of binding, $r = [DS_T]/C_P$, was held constant by using the above-described procedure. Under these circumstances, the data could be analyzed by equations applicable to the study of ordinary classical equilibria.[2] For this purpose we found convenient to use eq 2 since it allows[22] a simple presentation of the results (Figure 2A) and, at the same time, it is not limited by the need of using only data points collected under conditions of excess of one of the reactants, as it occurs in the Benesi−Hildebrand[23] analysis.

$$C_D C_P/\Delta A + \Delta A/\Delta\epsilon^2 = 1/(\Delta\epsilon K(r)) + 1/\Delta\epsilon(C_D + C_P) \qquad (2)$$

C_D and C_P are the total concentrations of dye and polymer respectively, $\Delta A = \text{Abs} - \epsilon_D C_D$ and $\Delta\epsilon = \epsilon_{DS} - \epsilon_D$. The two parameters $\Delta\epsilon$ and $K(r)$ were obtained from linear plots as that of Figure 2A by an iteration procedure where the term $\Delta A/\Delta\epsilon^2$ was initially neglected. Three iterations were sufficient to reach convergence. Titrations performed at different values of r in the range between 0.085 and 0.236 revealed that the values of $K(r)$ decrease by increasing r whereas the values of $\Delta\epsilon$ remain substantially constant. The average $\Delta\epsilon$ value of $-(3.3 \pm 0.3) \times 10^3\ M^{-1}\ cm^{-1}$ was used in the analysis of further experiments which were carried out by leaving r to change during titration. The variable r was evaluated by means of the relationship $r = \Delta A/(\Delta\epsilon C_P)$ in the absorbance method and by $r = \Delta A/(\Delta\varphi C_P)$ in the fluorescence method. The parameter $\Delta\varphi$ is defined as $\Delta\varphi = (F_{DS} - F_D)/C_D$, F_{DS} and F_D being the fluorescence signals of complex and dye, respectively.

Figure 2B shows a Scatchard curve with data points derived from three different sources: from a spectrofotometric titration (circles), from a spectrofluorimetric titration (triangles), and from a number of absorbance titrations each performed at constant value of r (squares). The value of r/[D] decreases linearly for $r \leq 0.25$. The positive deviation from linearity, which appears for $r > 0.25$, indicates site overlapping and is in agreement with an intercalation process where site exclusion occurs. The data have been analyzed according[24] to eq 3

$$r/[D] = K_{SC}(B - r) \qquad (3)$$

where B is a parameter associated with the site size and K_{SC} is the Scatchard binding constant. The slope and intercept of the plot of Figure 2B yield respectively $K_{SC} = 8.6 \times 10^4\ M^{-1}$ and $B = 0.30$. From these parameters one obtains the site binding constant $K = K_{SC}B = 2.6 \times 10^4\ M^{-1}$ and the site size $n = (1/B + 1)/2 = 2.2$.[25]

It should be mentioned that at low values of r (≤ 0.08) the Scatchard plots exhibit negative deviations from linearity. Similar behavior was observed for *E.coli* DNA/EB system[26] and was ascribed to cooperative binding. Other systems displaying positive cooperativity are Z-poly(dG-dC)/EB[27] and poly(dA)·poly(dT)/EB.[28] Scatchard plots exhibiting downward concavity were observed by Bresloff and Crothers[17] for several polynucleotide/EB systems. We have analyzed our data, including those at low values of r, by the Mc Ghee and Von Hippel[25] equation which takes into account the cooperativity parameter ω, but the fit was largely unsatisfactory. In addition, we have found that the deviations depend on the method employed, appearing for $r \leq 0.08$ in absorbance and for $r \leq 0.04$ in fluorescence experiments.

Finally, it should be noted that, in the range of the small r values, slight changes in ϵ_D and $\Delta\epsilon$ largely affect the values of both r and r/[D]. For these reasons the points deviating from linearity at low r values have not been included in the plot of Figure 2A.

Salt Effect in Equilibria. At neutral pH the ethidium bromide molecule bears a positive charge, therefore variations of the ionic strength of the solution are expected to influence the thermodynamics of the process of binding.[29]

The dependence of the equilibria on the concentration of added NaCl is shown in Figure 3 where log K_{SC} is plotted as a function of Log [Na^+] according to the equation:[30,31]

$$\log K_{SC} = \log K_0 + m'\psi \log[M^+] \qquad (4)$$

where K_0 is the binding constant free from the electrostatic contribution, m' is the number of phosphate residues per site and ψ is the extent of the counterion binding per phosphate. The slope $m'\psi$ represents thus the number of counterions released per occupied site.[32] The value of ψ increases as the distance between the phosphates decreases and for poly(A)·poly(U) is $\psi = 0.89$.[33] The slope of the line of Figure 3 is 1.01, this fact suggesting that each ethidium ion replaces one Na^+ counterion.

Kinetics. Kinetic experiments were performed by the stopped-flow technique at $\lambda = 480$ nm in the absorbance mode and at $\lambda_{exc} = 510$ nm in the fluorescence mode. Typical experiments are shown in Figure 4A. Most of the measurements were carried out with $C_P \gg C_D$ but some experiments were performed in excess of dye. The kinetic curves were appropriately fitted by two exponentials (Figure 4B), whose relaxation times τ_1 and τ_2

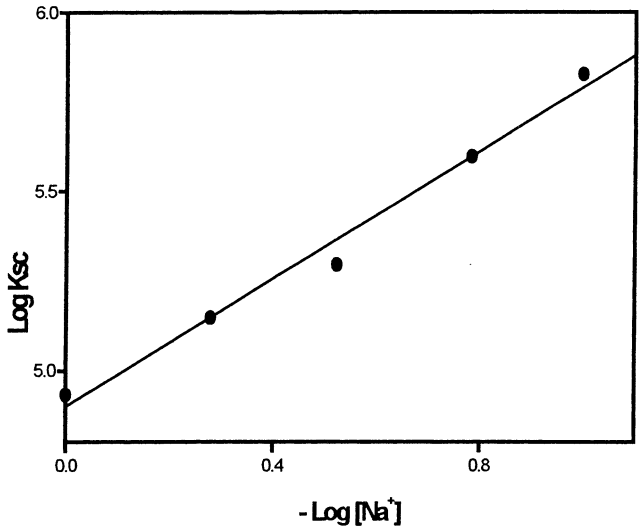

Figure 3. Dilogaritmic plot showing the dependence of the Scatchard binding constant K_{SC} on salt concentration. pH = 7.0, T = 25 °C. The ordinate value for $-\log[Na^+] = 0$ yelds the binding constant free from electrostatic contributions ($\log K_0 = 4.8$) whereas the slope $m'\psi = 1.01$ indicates that each ethidium ion replaces a sodium ion.

differ by less than 1 order of magnitude. Some experiments were performed changing C_P but keeping constant the value of r under conditions of polymer excess. It was found that the reciprocal of the slow relaxation time $1/\tau_2$ increases linearly with C_P even at high polymer concentrations, instead of tending to a plateau as expected for ordinary series or parallel mechanisms. It was also found that the relaxation times change by inverting the C_D/C_P ratio; actually, two experiments where ($C_P + C_D$) was kept constant at the value of 6×10^{-4} M gave, for $C_D/C_P = 11$, $1/\tau_1 = 207$ s^{-1}, and $1/\tau_2 = 31.5$ s^{-1}, whereas for $C_D/C_P = 0.017$ gave $1/\tau_1 = 132$ s^{-1} and $1/\tau_2 = 71.5$ s^{-1}. This observation clearly indicates that site overlapping plays an important role in the dynamics of the binding process; hence, the data were analyzed according to the procedure of Jovin and Striker,[3] even though most of the experiments have been performed under conditions of polymer excess, i.e., at relatively low r values.

The dependence of $(1/\tau_1 + 1/\tau_2)$ on the polymer concentration is linear (Figure 5A), whereas the dependence of $(1/\tau_1 \times 1/\tau_2)$ gives a parabola passing through the origin of the axes (Figure 5B). This behavior suggests the reaction scheme (cf. Appendix):

$$D + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} DS_I \tag{5}$$

$$S + DS_I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} DS_{II} + S \tag{6}$$

where step 5 represents the binding of ethidium to the RNA to form the complex DS_I, whereas step 6 represents an interstrand dye transfer with no dissociation of ethidium from either of the two strands.[2,4,7,10]

The Appendix shows that, according to this scheme, the sum of the reciprocal relaxation times is given by eq 7

$$1/\tau_1 + 1/\tau_2 = a + b[S] \tag{7}$$

where $a = k_{-1}$ and $b = k_1\alpha + k_2 + k_{-2}$.

The product of the reciprocal relaxation times is given by eq 8

$$(1/\tau_1)(1/\tau_2) = c[S] + d[S]^2 \tag{8}$$

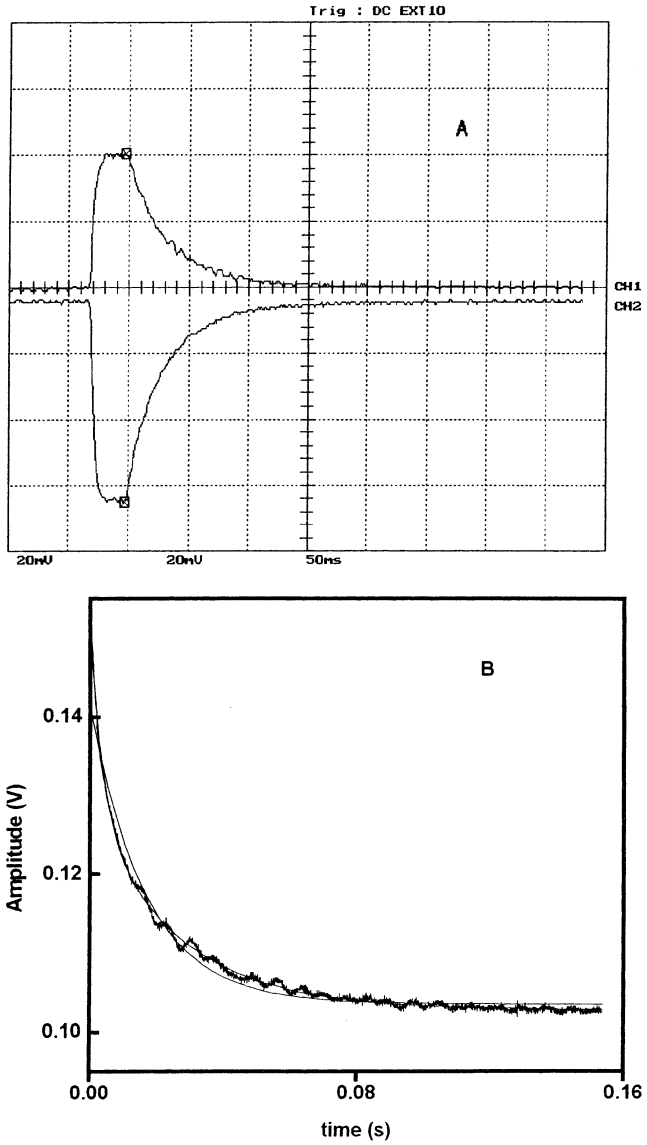

Figure 4. (A) Stopped-flow experiment for the poly(A)·poly(U)/EB system. The absorption (descending) and fluorescence (ascending) signals are detected simultaneously. (B) Mono- and biexponential analysis of a stopped-flow experiment in the absorbance mode performed with $C_D = 1 \times 10^{-5}$ M, $C_P = 2.9 \times 10^{-4}$ M, pH = 7.0, I = 1.0 M, and T = 25 °C. The biexponential fit superimpose exactly to the experimental points with $\tau_1 = 14$ ms and $\tau_2 = 63$ ms.

where $c = k_{-1} \times k_{-2}$ and $d = k_1\alpha(k_2 + k_{-2})$. Note that $\alpha = 1 - f'(r)[D]/[S]$ is near to unity in most experiments. The parameters of eqs 7 and 8 have been obtained by relative nonlinear least-squares procedures,[34] and the rate constants have been derived by suitable combinations of these parameters. Their values are $k_1 = 5.9 \times 10^4$ M^{-1} s^{-1}, $k_{-1} = 8.7$ s^{-1}, $k_2 = 1.8 \times 10^5$ M^{-1} s^{-1}, and $k_{-2} = 7.0 \times 10^4$ M^{-1} s^{-1}.

It should be noted that a cyclic mechanism differing from that above presented for the inclusion of the additional step

$$D + S \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} DS_{II} \tag{9}$$

was proposed for the DNA/EB system.[2,10] In this case the concentration dependence of $1/\tau_1 + 1/\tau_2$ stays linear, whereas that of $(1/\tau_1)(1/\tau_2)$ is still parabolic, but eq 8 should include an additional term e corresponding to $k_{-1} \times k_{-3}$. Hence, the possibility to distinguish between the two-step and the three-

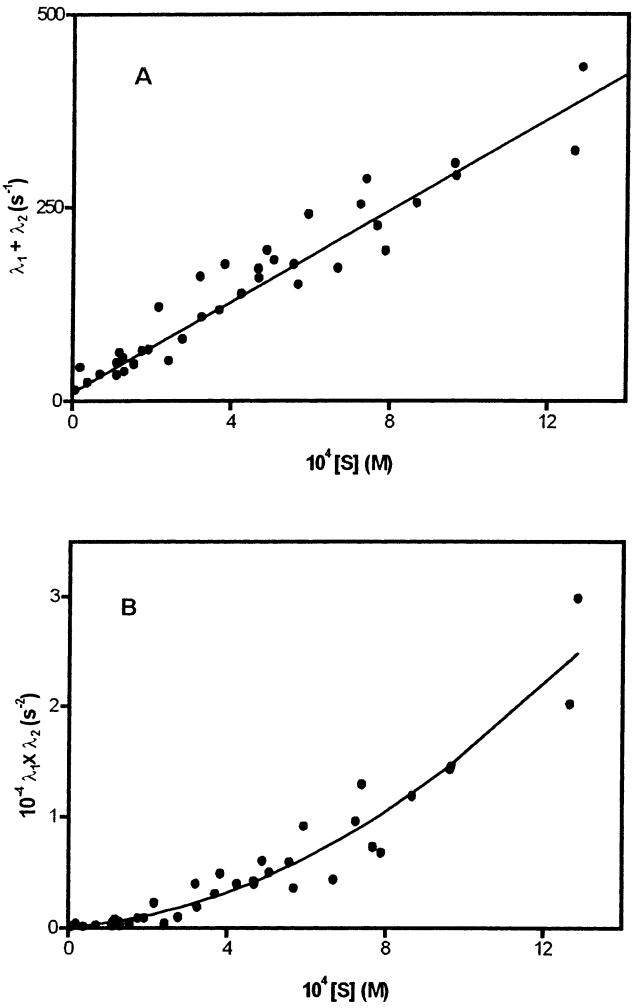

Figure 5. Analysis of the kinetic behavior of the poly(A)·poly(U)/EB system. pH = 7.0, I = 1 M, T = 25 °C. The site concentration [S] has been evaluated by multiplying the total polymer concentration C_P by the function $f(r)$ defined in the text. The rate constants have been derived by a combination of the kinetic parameters obtained from the two plots. (A) The calculated line is based on eq 7. (B) The calculated line is based on eq 8.

step mechanism lies in the value of the term e which should be definitely larger than zero in order to establish the occurrence of the three-step process. Our data, analyzed after addition of the term e to eq 8, yield $e = 9.6\ s^{-2}$, $c = 6.4 \times 10^5\ M^{-1}\ s^{-2}$, and $d = 1.4 \times 10^{10}\ M^{-2}\ s^{-2}$. To assess if the inclusion of e in eq 8 could provide a better representation of the experimental behavior, a student's t-test was performed on the parameter e. The student's t-test value associated with e was found to be lower than $t_{0.95}$. One can thus conclude that e is not significantly different from zero and therefore the two-step mechanism (steps 5 and 6) should be chosen to give the most appropriate representation of the experimental behavior.

The kinetic and the thermodynamic results were then compared by using the equation

$$K = k_1/k_{-1}(1 + k_2/k_{-2}) \qquad (10)$$

which links the individual equilibrium constants of steps 5 (k_1/k_{-1}) and 6 (k_2/k_{-2}) to the equilibrium constant K. Equation 10 yields $K = 2.6 \times 10^4\ M^{-1}$, which is in excellent agreement with the value obtained from the equilibria, thus providing a reliable check of the goodness of the results.

Discussion

Equilibria. The Scatchard analysis of the ethidium binding to double-stranded RNA from penicillium chrisogenum yields $K_{SC} = 4.7 \times 10^6\ M^{-1}$ at 25 °C in 0.1 M NaCl.[35] This value is somewhat higher than $K_{SC} = 6.7 \times 10^5\ M^{-1}$ here measured for poly(A)·poly(U)/EB under the same conditions (Figure 3). Our equilibrium parameters ($K_{SC} = 8.6 \times 10^4\ M^{-1}$ and $B = 0.3$ at 25 °C in 1 M NaCl) could be also compared with $K_{SC} = 11 \times 10^4\ M^{-1}$ and $B = 0.3$ obtained for poly(A)·poly(U)/EB at 19 °C in 1 M $NaNO_3$.[17] Whereas the B values are identical, our binding constant is lower. We ascribe the difference to the higher temperature of our experiments since the binding of ethidium to poly(A)·poly(U) is an exothermic process[19,36] with $\Delta H° = -9$ kcal mol^{-1} or -7.2 kcal mol^{-1}.

We observe as well that $K_{SC} = 2.5 \times 10^6\ M^{-1}$, measured for the E.coli tRNA/EB system[12] at 25 °C and $I = 0.056$ M (imidazole + $MgCl_2$), compares with our value of $1.3 \times 10^6\ M^{-1}$ for poly(A)·poly(U)/EB extrapolated to the same ionic strength with the help of the plot of Figure 3. The above comparisons indicate that the affinity of ethidium for RNA is rather indipendent of the ribonucleotide origin.

A comparison between the affinity of RNA/EB and DNA/EB systems has been made by Bresloff and Crothers.[17] These authors did not found large differences in the binding strength when changing from RNA to DNA. This observation contrasts with results from the study of the binding strength of EB to the oligonucleotides ($rGA_5C + rCU_5G$) and ($dA_5G + dCT_5G$) where the affinity of the dye for the ribo-form is higher than that for the deoxyribo-form.[18]

The dependence of the equilibrium constant on the sodium chloride concentration (Figure 3) reveals that polyelectrolyte effects influence considerably the interactions of EB with poly(A)·poly(U). Similar effects have been observed in DNA/dye systems as well,[32] and ascribed to cation displacement from strands upon dye binding. Actually, the prediction of an important fraction of counterions externally bound to the polymer chain, made by a number of authors,[37−39] has been confirmed for sodium by ^{23}Na-NMR studies.[40−42]

Kinetics. Whereas the static measurements provide information about the overall binding process, the kinetic results enable us to establish that the interaction of ethidium with poly(A)·poly(U) gives rise to two different complexes and to evaluate their relative populations.

Owing to the lack of kinetic investigations on RNA/EB systems, the kinetic data here presented can be compared only with those from DNA/EB systems.[2,7,10] Our series two-step model should be considered as a special case of the cyclic model (holding for DNAs/EB) where the exchange rate of step 9 r_3 is much lower than r_1 and r_2 (cf. Appendix). The causes of this difference are not clear but they should probably lie in the structural differences existing between the A and B forms of nucleic acids. In particular, the interaction of the phenyl residue with the wide and narrow groove might be responsible for the different kinetic behavior of the two complexes. It is however worth of notice that, for DNA/EB as well, step 9 gives only a minor contribution to the rate of the overall process of binding. Actually, the data of Ryan and Crothers[7] for KT-DNA/EB yield $r_3/r_1 = 0.19$ as the rate ratio for the exchange reactions 9 and 5 whereas the data at 25 °C of Meyer Almes and Pörschke[10] yield $r_3/r_1 = 0.15$ for the same system, and $r_3/r_1 = 0.075$ for the poly[d(A−T)]/EB. In our case, a value of r_3/r_1 less than 0.1 can made the detection of step 9 quite difficult, due to the relatively high scatter of the data.

The reactivity of poly(A)·poly(U)/EB could be compared with that of DNA/EB by making the reasonable hypothesis that our step 5 corresponds to the faster of the two parallel steps of the cyclic scheme holding for DNA/EB. Hence, our value of k_1 turns out to be 24 times lower than that measured by Ryan and Crothers[7] for DNA/EB in 1 M $NaNO_3$ and 12 times lower than that measured by Meyer-Almes and Pörschke[10] in 0.1 M $NaClO_4$. The last factor could be reduced by 1 order of magnitude by considering that a change of ionic strength from 0.1 to 1 M would presumably reduce the reaction rate by a factor of 10, as observed for the equilibria. The complex dissociation rate constant k_{-1}, which is expected to be not sensitive to changes of ionic strength, is 20 times lower than that quoted for DNA/EB in 1 M $NaNO_3$, 4.5 times lower than that in 0.1 M $NaClO_4$ and has about the same value as that of the poly[d(A−T)] system in 0.1 M NaCl.[9] Concerning the direct transfer step (reaction 6), we observe that both (the forward and the reverse) reaction constants are similar to those evaluated for DNA/EB[7] at $I = 1$ M, but lower than those obtained[10] at $I = 0.1$ M. Normalization for the ionic strength effect results in a reduction of the values of both rate constants of the direct exchange step by a factor of 10 which brings the values of k_2 and k_{-2} for DNA/EB near to those for poly(A)·poly(U)/EB.

The formation of two complexes is not in contrast with spectral changes (Figure 1B) and has been confirmed by the kinetic behavior of the system. Ryan and Crothers,[7] explained the analogous finding in the DNA/EB system by assuming that DNA offers to the dye two classes of binding sites, respectively associated to a weak mode of binding (fast effect) and to a strong intercalative mode of binding (slow effect). Meyer-Almes and Porschke[10] observed two complexes as well in the poly[d(A−T)]/EB system and concluded that their formation could not be due to a heterogeneity of binding sites, but to intercalation of the phenanthridinium with the location of the phenyl residue respectively in the narrow and in the wide groove.

A recent investigation[15] on the kinetics of intercalation of proflavine into double-helical poly(A) has revealed that two nonelectrostatic complexes are formed. In this case the difference between the two bound forms could not be ascribed to groove-involvement since double stranded poly(A) exhibits only a single groove. Therefore, the relatively fast formation of a preintercalated complex, followed by a slower step where the dye insertion is completed, has been advanced to explain the kinetic behavior. Preintercalation was suggested as well by Zimmerman[43] and Schelhorn et al.[44] to interpret the behavior of acridine binding to DNA.

Pörschke[11] has studied the dynamics of the DNA/EB system by a new electrooptical method and demonstrated that the fraction of outside complex is minority, that two different complexes are formed at different rates and that the ethidium is intercalated in both complexes.

We divide the kinetic investigations on the DNA/EB system in two groups: a group where two relaxation effects have been observed and a group where only one relaxation has been found. Among these we cite the interesting papers of MacGregor et al.[9,48] who measured a single kinetic effect with a second-order time constant lower than the diffusional limit by about 3 orders of magnitude. Moreover, this constant changes linearly with the reciprocal viscosity. The authors interpret their findings on the basis of a two-step mechanism involving a fast preequilibrium between two conformation isomers of DNA, of which only one is reactive. It is assumed that the reactive species, while it binds EB at diffusion controlled rate, is minority, its molar fraction being 10^{-3}, so that the observed rate constant is reduced by about 3 orders of magnitude with respect to the diffusional limit.

An alternative mechanism, in principle able to explain the low binding rates, which is favored by other authors[10] involves, as the first step, the inset of a fast binding equilibrium where an electrostatic DNA-ethidium complex is formed.

The two outlined mechanisms could not be kinetically distinguished since the intermediate concentrations are low compared to that of the other species.[48,11] Both interpretations can in principle be applied to our system.

Concerning the second kinetic effect observed in this study it should be noted that the range of reactant concentrations of our experiments exceeds by about an order of magnitude that of the works of McGregor et al.[9,48] Our conditions are therefore more appropriate in order to detect step 6. One can calculate, by using the values of the rate constants of this work that for $C_P = C_D = 2 \times 10^{-5}$ M (corresponding to conditions of the above cited papers) the contribution of step 6 to the overall intercalation process is only 30%.

Additionally, one should mention that the number of the observable kinetic effects depends on the employed technique. Relaxation methods allow the observation of normal modes of reaction rather than individual coupled reactions. In case of P-Jump the relaxation amplitude of each normal mode is directly proportional to the relevant reaction volume $\Delta V°$. Referring to scheme 5−6, the reaction volume associated to the second effect is[49] $\Delta V_{II}° = \Delta V_5° + (C_{22}/C_{12})\Delta V_6°$, where $\Delta V_5°$ and $\Delta V_6°$ are the reaction volumes of steps 5 and 6, whereas C_{22}/C_{12} could be evaluated[49] with the help of eq A14. If the above linear combination is such that $\Delta V_{II}°$ takes a sufficiently small value, the second effect could well vanish.

Finally, we note that even in stopped-flow experiments, where, owing to larger amplitudes, the signal-to-noise-ratio is more favorable than in relaxation experiments, the differences between the curves calculated by the one-exponential and two-exponential analysis is small (Figure 4B). Therefore, it is not surprising that in relaxation experiments, where the amplitudes are smaller and the noise presumably higher, the second effect could remain undetected.

Although simpler, owing to the suppression of step 9, the kinetic behavior of the poly(A)·poly(U)/EB system displays important analogies with that of DNA/EB systems. These analogies hints at the idea that the interaction of ethidium with RNA double-strands, presents essentially the same characteristics observed for the interaction with DNA. However the lack of studies with other sequences could limit our conclusions. In this context experiments with different dsRNAs are highly desiderable.

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Appendix

The kinetic behavior of a multiple equilibrium system, constituted by R coupled and thermodynamically independent elementary reactions, can be expressed by eq A1:

$$-d\boldsymbol{\zeta}/dt = \mathbf{r} \times \mathbf{g} \times \boldsymbol{\zeta} \qquad \text{(A1)}$$

where $\mathbf{r}$ is a diagonal matrix whose elements are the exchange rates of the R reactions, and $\mathbf{g}$ is a $(R \times R)$ symmetric matrix whose elements are defined according to the Castellan's paper.[45] $\boldsymbol{\zeta}$ is a column vector whose elements indicate the extent of reaction of the R elementary steps.

Once the elements of the $R \times R$ matrix $\mathbf{r} \times \mathbf{g}$ have been defined according to a given reaction scheme, the dynamical behavior of the system can be described by solving the eigenvalue problem, which leads to the system of homogeneous linear equations:[49]

$$(\mathbf{r} \times \mathbf{g} - \lambda \mathbf{I})\mathbf{C} = 0 \tag{A2}$$

The eigenvalues λ_k of the matrix $\mathbf{r} \times \mathbf{g}$ coincide with the reciprocal relaxation times. **I** is the identity matrix, whereas **C** are column vectors associated to λ_k. A nontrivial solution to eq A2 exists only if the determinant of the matrix $(\mathbf{r} \times \mathbf{g} - \lambda_k \mathbf{I})$ is zero.

The reaction scheme for poly(A)·poly(U)/EB interaction involves two coupled thermodynamically independent reactions

$$\mathrm{D} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{DS_I} \tag{A3}$$

$$\mathrm{S} + \mathrm{DS_I} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{DS_{II}} + \mathrm{S} \tag{A4}$$

The elements of the diagonal matrix **r** are

$$r_1 = k_1 \times [\mathrm{S}] \times [\mathrm{D}] = k_{-1} \times [\mathrm{DS_I}] \tag{A5}$$

$$\mathrm{r}_2 = k_2 \times [\mathrm{S}] \times [\mathrm{DS_I}] = k_{-2} \times [\mathrm{S}] \times [\mathrm{DS_{II}}] \tag{A6}$$

where the terms in brackets are equilibrium concentrations. For overlapping sites, the elements of the (2×2) matrix **g** are:

$$g_{11} = f'(r)/[\mathrm{S}] + 1/[\mathrm{D}] + 1/[\mathrm{DS_I}] \tag{A7}$$

$$g_{12} = g_{21} = -1/[\mathrm{DS_I}] \tag{A8}$$

$$g_{22} = 1/[\mathrm{DS_I}] + 1/[\mathrm{DS_{II}}] \tag{A9}$$

eq A7 has been proposed by Jovin and Striker[3] in replacement of $g_{11} = 1/[\mathrm{S}] + 1/[\mathrm{D}] + 1/[\mathrm{DS_I}]$ that would holds for ordinary reactions.

The elements of the (2×2) $\mathbf{r} \times \mathbf{g}$ matrix become

$$r_1 g_{11} = k_1(1 - f'(r) \times [\mathrm{D}]/[\mathrm{S}])[\mathrm{S}] + k_{-1} \tag{A10}$$

$$r_1 g_{12} = -k_{-1} \tag{A11}$$

$$r_2 g_{21} = -k_2 \times [\mathrm{S}] \tag{A12}$$

$$r_2 g_{22} = (k_2 + k_{-2}) \times [\mathrm{S}] \tag{A13}$$

The site concentration could be evaluated from the total polymer concentration C_P as $[\mathrm{S}] = f(r)C_\mathrm{P}$.[25] The varible $f(r)$ and its derivative $f'(r)$ are defined in the paper of Jovin and Striker.[3] The reciprocal relaxation times are linked to concentrations and rate constants by eq A14.

$$\det(\mathbf{r} \times \mathbf{g} - \lambda\, \mathbf{I}) = 0 \tag{A14}$$

According to this equation the sum of the eigenvalues is given by the relationship:

$$\lambda_1 + \lambda_2 = r_1 g_{11} + r_2 g_{22} \tag{A15}$$

and the product is given by the relationship:

$$\lambda_1 \times \lambda_2 = r_1 r_2 (g_{11} g_{22} - g_{12} g_{21}) \tag{A16}$$

Introduction of eqs A10−A13 in eq A15 yields eq 7, whereas introduction of eqs A10−A13 in eq A16 yields eq 8. Note that in the text λ_1 and λ_2 are indicated as $1/\tau_1$ and $1/\tau_2$, respectively.

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