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Stopped-Flow Kinetic Studies on the Interaction between Echinomycin and DNA†

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ABSTRACT: The kinetics of association between the quinoxaline antitumor antibiotic echinomycin and DNA have been studied by stopped-flow methods. With natural DNAs, the reaction profile is completely described by a single exponential, the time constant for which varies linearly with the DNA concentration. This bimolecular rate constant is similar for both calf thymus and *Micrococcus lysodeikticus* DNA ($k = 6 \times 10^4$ M^{-1} s^{-1} at 25 °C, $I = 0.01$) and is probably dominated by interaction with relatively weak but abundant binding sites from which the antibiotic dissociates fairly quickly. The observed single exponential suggests a molecular mechanism of binding in which both chromophores of the antibiotic become intercalated simultaneously rather than sequentially; no transition from a mono-intercalated state to a bis-intercalated state could be detected. The reaction is slowed by a factor of about 3 on raising the salt concentration from $I = 0.01$ to $I = 0.5$. Binding to poly(dA-dT) is also described by a single exponential, the time constant for which is about 3 times faster than that seen with natural DNAs. By contrast, the interaction with poly(dG-dC) requires two exponentials for a proper description, the faster of which is similar to that seen with natural DNAs. This may reflect the initial interaction of the antibiotic with two types of sequences, tentatively identified as GpC and CpG, from which it dissociates at very different rates. The differences in kinetic behavior may be explicable on the basis of an alternating B structure for poly(dA-dT) and a more classical B form for poly(dG-dC).

The quinoxaline group of antibiotics, of which echinomycin (Figure 1) is the best known member, are highly active against Gram-positive bacteria, viruses, and a variety of experimental tumors (Katagiri et al., 1975). Their biological properties have been attributed to their ability to bind to DNA (Ward et al., 1965; Sato et al., 1967; Waring & Makoff, 1974). All of them seem to interact specifically with DNA (not RNA) via the process of bifunctional intercalation (Waring & Wakelin, 1974; Lee & Waring, 1978; Waring & Fox, 1983). Detailed studies with echinomycin and a variety of natural and synthetic DNA species have revealed differences in the apparent binding constants ranging over more than 1 order of magnitude (Wakelin & Waring, 1976). While there is no straight-forward correlation between the measured binding constants and

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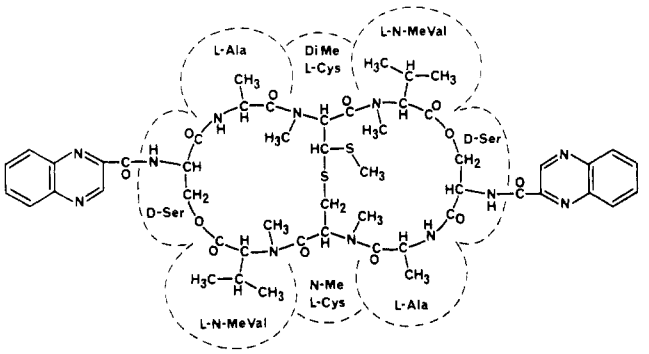


FIGURE 1: Structure of echinomycin.

the gross base composition of the DNA, the antibiotic displays a distinct tendency to bind more tightly to those DNAs rich in guanine and cytosine (G+C) residues.

We have previously shown (Fox et al., 1981) that the detergent-induced dissociation of echinomycin from the synthetic DNAs poly(dG-dC) and poly(dA-dT) is adequately described by a single exponential whereas its dissociation from natural DNAs requires three exponentials for a complete description. This behavior is similar to that seen with actinomycin (Müller & Crothers, 1968; Krugh et al., 1979) and seems to be explained by dissociation of the antibiotic from different classes of intercalative binding sites. Accordingly, these studies have provided uniquely informative insights into the character of sequence preferences in the binding of the antibiotics to DNA.

In this paper, we describe investigations with stopped-flow kinetics to probe the nature of the association reaction between echinomycin and DNA. Previous studies with actinomycin, which shares a number of structural features in common with the quinoxaline group of antibiotics, have shown that its association reaction profile is very complicated, requiring at least five exponentials to provide a complete description (Müller & Crothers, 1968; Bittman & Blau, 1975). Of the five rate constants, the fastest two appeared to vary with DNA concentration and thus might correspond to the initial bimolecular antibiotic–DNA association reaction(s). With echinomycin, we previously reported observing a slow component in its reaction with DNA, detectable by direct mixing and responsible for about 20% of the total absorbance change (Fox et al., 1981). As the earlier workers suggested for actinomycin, we tentatively attributed this slow, DNA concentration independent effect to a conformational change in the antibiotic–DNA complex, subsequent to the initial bimolecular reaction (Fox et al., 1981).

The experiments reported here were undertaken with the objective of properly characterizing the echinomycin–DNA association reaction, with particular reference to three questions. Does the reaction profile display the same features of complexity as seen in the binding of actinomycin, to match the known complexity of the dissociation reactions seen with both antibiotics? Is it possible to observe the two putative steps of a bis-intercalation process, i.e., intercalation of one chromophore followed by intercalation of the other, or does the interaction proceed to all intents and purposes as a single concerted process involving simultaneous insertion of both chromophores between the DNA base pairs? Are there indications of "shuffling" reactions whereby bound antibiotic molecules migrate from one binding site to another in search of optimizing ligand–polynucleotide interactions? The results have provided some surprises and lead us to propose a kinetic model for the interaction between the antibiotic and its macromolecular receptor, including some insight into the means by which echinomycin attains binding to its preferred sites.

Materials and Methods

Chemicals. Except where otherwise stated, all experiments were conducted in Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]–NaOH buffer, pH 7.0, ionic strength 0.01, designated 0.01 SHE. A stock solution containing 0.2 M Hepes, 1 mM EDTA (ethylenediaminetetraacetic acid), and 0.94 M NaCl was adjusted to pH 7.0 and stored frozen. This stock solution was diluted 100-fold, with no significant change in pH, to give a resultant ionic strength of 0.01. Reagent-grade water from a Millipore Milli-Q2 system was used throughout. Echinomycin was a gift from Drs. H. Bickel and K. Scheibli, Ciba-Geigy Ltd., Basel, Switzerland.

Calf thymus DNA (highly polymerized sodium salt, type I) was purchased from Sigma Chemical Co. For *Micrococcus lysodeikticus* DNA, 5 g of lyophilized cells was purchased from Sigma Chemical Co., and the DNA was extracted by a modification of the method of Marmur (1961) employing a Pronase digestion step, as described in Lee & Waring (1978). Both these natural DNAs were dissolved in 0.01 SHE (3 mg/mL) and sonicated for about 5 min at 0 °C with the exponential probe of an MSE 150W ultrasonic disintegrator at the highest power level, in order to reduce their viscosity. Poly(dG-dC) and poly(dA-dT) were purchased from the Boehringer Corp., dissolved in 0.01 SHE, and used without further purification. Nucleic acid concentrations are expressed with respect to nucleotides on the basis of the following values for $E(P)_{260}$: calf thymus DNA, 6600; *M. lysodeikticus* DNA, 6300; poly(dA-dT), 6700; poly(dG-dC), 7100.

Because of its extremely low solubility in aqueous solvents (≤ 5 μM), solutions of echinomycin in 0.01 SHE were prepared by shaking an excess of the solid antibiotic with buffer for a few hours and then filtering off the excess solid. The concentration in solution was estimated from the absorbance at 245 nm on the basis of a value for E_{245} of 48 200 (Waring et al., 1975). For those experiments performed at higher ionic strength, the solutions were first prepared in 0.01 SHE, and sufficient sodium chloride was then added, with no significant change in pH, to yield an ionic strength of 0.5.

Stopped-Flow Apparatus. Kinetic experiments were performed with a Hi-Tech SF3 stopped-flow spectrometer and a standard-volume SF-31/10 flow module with 10-mm path length (Hi-Tech Instruments, Salisbury, Wiltshire, England). The dead time was 7 ms. It was necessary to use this long path length cell with its consequential longer dead time because of the low solubility of the antibiotic. The Hi-Tech LS34 xenon arc light source and power supply were used without modification. The beam was passed through a Bausch & Lomb high-intensity monochromator via light guides to the mixing cell, with the photomultiplier direct mounted. The signal from the photomultiplier was stored on a Datalab DL905 transient recorder and displayed on a Tektronix Model 5103N oscilloscope. Data were extracted from the transient recorder as eight-bit parallel binary numbers on paper tape, with a Data Dynamics D1133 punch, for subsequent computer analysis.

The photomultiplier was typically offset at about 6 V, and the transient recorder set to 100-mV full-scale deflection. The recorder was programmed to collect 1024 data points with a time base, depending upon the experimental conditions, ranging from 0.1 to 10.0 ms per datum point. The double time base feature of the recorder was used to optimize the amount of data collected within the initial period when the signal was changing most rapidly as well as to provide an equilibrium value needed for numerical analysis of the experimental curves.

Routinely, kinetic experiments were conducted by mixing equal volumes of solutions of echinomycin (4–5 μM) and DNA (0.1–1.0 mM) so as to yield a final drug concentration of 2–2.5 μM and a nucleotide to antibiotic molar ratio (P/D) greater than 20. The change in absorbance at 320 nm was recorded. Under these conditions, the DNA concentration remains essentially constant throughout the reaction, and the association process approximates to a pseudo-first-order reaction. Generally, each reaction was repeated 10 times, and the results are presented as an average of all determinations along with their standard errors.

Analysis of Kinetic Data. The data were analyzed with the aid of a program written by Johnson & Schuster (1974) according to a nonlinear least-squares Gauss–Newton method. Dr. M. Johnson of the Department of Biochemistry, University of Virginia, Charlottesville, VA, kindly provided a copy of his Fortran IV computer program, which was installed on the Cambridge University IBM 3081 computer. The program was initially developed for the analysis of temperature-jump relaxation data (Wakelin & Waring, 1980) and allows decomposition of up to three exponentials according to

$$A_t = A_1e^{-t/\tau_1} + A_2e^{-t/\tau_2} + A_3e^{-t/\tau_3}$$

where τ_1, τ_2, and τ_3 are the time constants of the decay and A_1, A_2, and A_3 are their respective amplitudes. The program provides several statistical parameters for checking that the data have been properly handled and tests for overdetermination. Its use has been described previously by Fox et al. (1981). The root-mean-square values of the residuals to the best fits were always indistinguishable from the noise inherent in the data, commonly about 2–3 mV.

Determination of Thermodynamic Parameters. Throughout these investigations, the energy of activation ΔE and the entropy of activation ΔS^* have been estimated from analyses of Arrhenius plots. In these plots, the slope yields $-\Delta E/R$, and the intercept on the axis of ln $(1/\tau)$ corresponds to ln A, where A is the preexponential factor in the equation $k = Ae^{-\Delta E/(RT)}$. The value of A provides an indication of steric and entropic factors involved in the activation process. The entropy of activation is derived from the value of A by assuming that $A = (kT/h)e^{\Delta S^*/R}$, where k is the Boltzmann constant and h is the Planck constant.

Results

In preliminary experiments to investigate the formation of complexes between 2.5 μM echinomycin and 200 μM calf thymus DNA at 20 °C, it was found that the optical change (ΔOD = 0.009) was essentially complete by 0.5 s with no longer components detectable up to 10 s. A representative curve is shown in Figure 2A. The reaction profile was adequately described by a simple (single) exponential process, and attempts to resolve it into two or more exponentials failed. It therefore appears that, over the time scale accessible to the stopped-flow apparatus, the interaction between echinomycin and calf thymus DNA associated with an absorbance change at 320 nm behaves as a simple one-step process. This observation stands in contrast to the much more complex profile seen with actinomycin (Figure 2B). In order to investigate the molecular events underlying this absorbance change, a series of experiments were performed in which the reaction kinetics were measured at various DNA concentrations and temperatures. Under all conditions, the reaction profile was adequately described by a simple exponential decay, except possibly at the lowest DNA concentration investigated where deviations from a pseudo-first-order process can be expected because the concentration of free binding sites changes significantly during the course of reaction. The results of these experiments showed that the rate constant for this reaction is linearly dependent on the DNA concentration at all the temperatures investigated. The bimolecular rate constants obtained from the gradients of the least-squares lines fitted to the points were as follows: at 25 °C, (6.2 ± 1.0) × 10^4 M^{-1} s^{-1}; at 30 °C, (10.0 ± 1.0) × 10^4 M^{-1} s^{-1}; at 40 °C, (18.2 ±

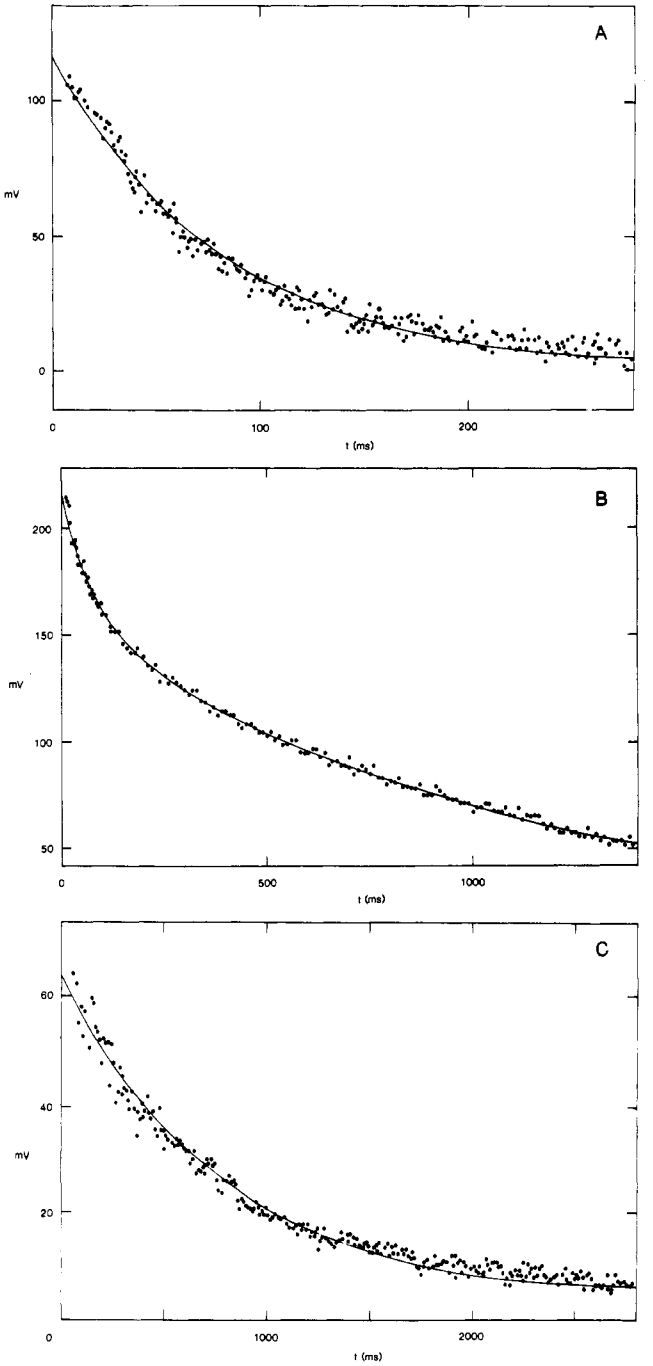


FIGURE 2: Typical kinetic traces showing the binding of echinomycin and actinomycin to DNA. In each trace the ordinate represents the amplitude of the optical signal relative to the final equilibrium value set to 0 mV. (A) Interaction of echinomycin (2.5 μM) with 200 μM calf thymus DNA, I = 0.01, at 25 °C. The single exponential curve fitted to the points has a time constant (τ) of 83 ms and an amplitude of 117 mV. (B) Interaction of actinomycin D (3 μM) with 200 μM calf thymus DNA, I = 0.01, at 25 °C. The curve fitted to the points corresponds to three exponentials characterized by time constants of 63 ms, 437 ms, 2.0 s with amplitudes of 48, 60, and 110 mV, respectively. (C) Interaction of echinomycin (1.8 μM) with 100 μM calf thymus DNA, I = 0.5, at 15 °C. The single exponential curve fitted to the points has a time constant (τ) of 802 ms and an amplitude of 62 mV.

Table I: Kinetic and Thermodynamic Parameters for the Binding of Echinomycin to Natural DNAs and Poly(dA-dT) [a]

	$k_{20°C}$ (M^{-1} s^{-1})	ΔE (kcal mol^{-1})	ln A	$\Delta S^{\ddagger}$ (eu mol^{-1})	K (0) (μM^{-1})	K_{calcd} (μM^{-1})
calf thymus DNA, I = 0.01	(5.1 ± 0.8) × 10^4	11.9 ± 0.8	31.2 ± 1.4	2.5	0.55	3.0
calf thymus DNA, I = 0.5	(1.7 ± 0.2) × 10^4	11.9 ± 0.9	30.1 ± 1.6	0.3	0.104	0.44
M. lysodeikticus DNA	(4.5 ± 0.2) × 10^4	11.3 ± 0.4	30.2 ± 0.7	0.5	3.10	6.6
poly(dA-dT)	(9.7 ± 0.8) × 10^4	15.0 ± 1.4	36.9 ± 2.4	13.9	0.31	2.43

[a] The observed values for the equilibrium binding constant $K(0)$ are taken from Wakelin & Waring (1976). K_{calcd} represents the value calculated for the binding constant from the kinetic parameters, as described in the text.

2.8) × 10^4 M^{-1} s^{-1}; at 45 °C, (24.5 ± 3.8) × 10^4 M^{-1} s^{-1}; all expressed in terms of total DNA nucleotides. At each temperature, the least-squares line fitted to the data points passes through the origin, within experimental error, suggesting that the dissociation reaction is very much slower, consistent with the dissociation kinetics previously reported (Fox et al., 1981). From these data, it is possible to construct Arrhenius plots to investigate the origins of the temperature dependence of the reaction. This can be done in one of two ways. The first method is to construct an Arrhenius plot of the logarithm of the true bimolecular rate constants against the inverse of temperature, which yielded a straight line with slope (6.4 ± 0.6) × 10^3 and intercept 32.6 ± 1.9. From the slope of this plot, the activation energy is calculated to be 12.8 kcal mol^{-1}. An alternative, less precise, method of analyzing the data is to calculate separate Arrhenius plots for each of the DNA concentrations employed. These each gave the same value for the activation energy, derived from their slopes, and a representative example is shown in Figure 3A. Since both approaches yield the same result, and the observed reaction appears to represent the bimolecular association process; in all further experiments [except those with poly(dG-dC)], a linear relationship between the observed rate constants and DNA concentration was assumed, and experiments were conducted at a standard DNA concentration (100 μM) in order to optimize the signal-to-noise ratio and to conserve valuable materials.

The equilibrium constant for echinomycin–DNA binding is known to decrease as the salt concentration is raised (Wakelin & Waring, 1976). It has also been shown that the rates of dissociation become faster at higher ionic strengths, although the increase in dissociation rates is less marked than the variation in the binding constants (Fox et al., 1981). It was therefore predicted that the association reaction between echinomycin and DNA might become slower at elevated ionic strengths. To test this prediction, a series of stopped-flow experiments were performed at I = 0.5. Again, the reaction profiles observed were well fitted by single exponentials, and a representative curve is shown in Figure 2C. At each temperature investigated, the reaction was found to proceed 2–3 times slower at the higher ionic strength, as anticipated (Fox et al., 1981). Figure 3A shows the Arrhenius plots calculated from these single exponential fits to the data (all results at 100 μM DNA concentration). The slopes of the plots at the two ionic strengths are the same, within experimental error, showing that the activation energy does not change significantly with ionic strength and suggesting that the activated states may be the same under both conditions. The parallel displacement of the curves clearly indicates that the entropies of activation must be different at the two ionic strengths over the temperature range investigated. This could be due to differences in the number of water molecules being displaced from the DNA helix prior to drug binding or perhaps to an ionic effect resulting from the increased phosphate–phosphate distance on extending the DNA (Record et al., 1976).

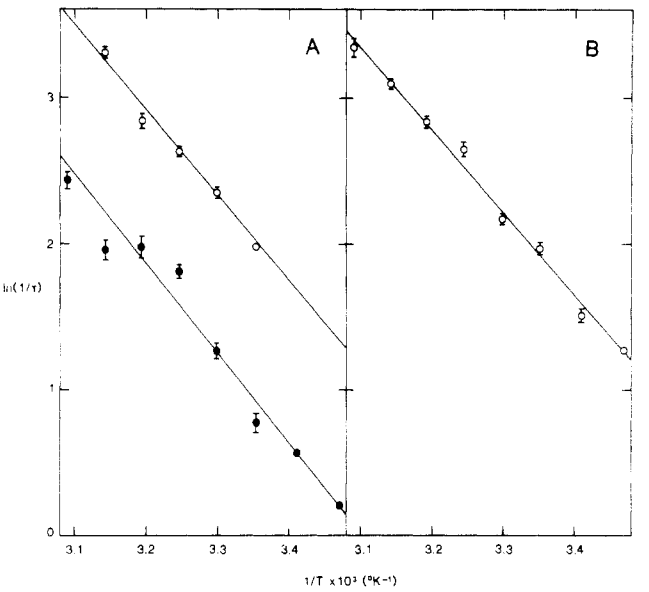


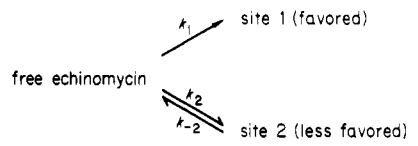
FIGURE 3: Arrhenius plots showing the variation with temperature of pseudo-first-order rate constants for the interaction between echinomycin and natural DNA species. The DNA concentration was 100 μM throughout: (○) I = 0.01; (●) I = 0.5. The left panel shows data obtained with calf thymus DNA. The least-squares lines fitted to the points are characterized by the following parameters: at I = 0.01, intercept = 22.0 ± 1.4, slope = (5.9 ± 1.4) × 10^3; at I = 0.5, intercept = 20.9 ± 1.6, slope = (6.0 ± 0.5) × 10^3. The right panel shows data obtained with *M. lysodeikticus* DNA. The least-squares line fitted to the points has a slope = (5.7 ± 0.2) × 10^3 and an intercept = 20.9 ± 0.7.

Echinomycin binds about 6 times more tightly to *M. lysodeikticus* DNA than to calf thymus DNA at I = 0.01 (Wakelin & Waring, 1976), and its dissociation kinetic profile is distinctly different (Fox et al., 1981). To complete the comparison between DNAs and investigate the effects of base composition on the rate of the forward reaction, the kinetics of interaction with *M. lysodeikticus* DNA were also investigated at various temperatures. Once more, the data were adequately described by single exponential curves. The rate constants derived from them are presented in the form of an Arrhenius plot in Figure 3B. The parameters resulting from this analysis (see Table I) are very similar to those determined for calf thymus DNA, suggesting that the overall bimolecular reaction observed is not greatly affected by the gross base composition of natural DNA. This result is slightly surprising in view of the pronounced sequence selectivity of the antibiotic, if only because it seems likely that different energies would be required to unstack each of the various dinucleotide sequences. However, the observed bimolecular rate constant will actually be a composite of the various microscopic constants for each of the possible sequences with which the ligand interacts. It is also possible that the sequences to which the antibiotic initially binds may not actually be the preferred sites and that the rearrangement on to the small number of preferred sites is not associated with a detectable absorbance change. If this is the case, than for both natural DNAs the

observed bimolecular rate constant will be dominated by interaction with the most abundant sites and not necessarily the most tightly binding ones.

In an effort to detect any variations in the rate of reaction with different DNA sequences that might not be evident when natural DNAs are used, the stopped-flow kinetics of interaction between echinomycin and synthetic defined-sequence polynucleotides were investigated. With 100 μM poly(dA-dT), the reaction profiles were all adequately described by a single exponential process; however, at each temperature investigated the reaction was faster than that observed with the two natural DNAs. When the rate constants measured at the various temperatures with this synthetic polynucleotide are plotted in the form of an Arrhenius plot, this yields a straight line with a slope of $(7.5 \pm 0.7) \times 10^3$ and intercept of 27.7 ± 2.4. The activation energies and entropies calculated for the reaction with poly(dA-dT) are significantly larger than those determined for natural DNAs (Table I) and are considered further under Discussion. Although it was not possible to conduct an exhaustive series of investigations into the concentration dependence of this reaction, it was observed to become slower at lower polynucleotide concentrations as expected.

By contrast, the stopped-flow kinetic profiles observed for the interaction between echinomycin and poly(dG-dC) were not adequately described by a single exponential curve but, instead, required two exponentials for their complete description. With 100 μM polynucleotide at 20 °C, the two time constants were separated by a factor of 5 with about 80% of the total absorbance change in the faster reaction. This observation is somewhat surprising since the interaction with all other DNAs investigated proved totally compatible with a single exponential. Two hypotheses can be advanced to account for this difference. The first possibility is that subsequent to the initial (bimolecular) interaction with poly(dG-dC), the antibiotic molecule undergoes a further rearrangement causing a change in its spectral properties. This seems rather unlikely since no such rearrangement was detected with any of the other DNAs and also the UV absorption spectrum of the antibiotic bound to poly(dG-dC) is practically indistinguishable from that of echinomycin bound to natural DNAs. The other suggestion derives from the idea that initially the drug interacts with both GpC and CpG sequences on the polynucleotide. This in itself would not be sufficient to produce a two-exponential reaction profile, since the interaction would result in a single exponential decay with a rate constant given by the sum of the individual rate constants for interaction with the two nucleotide sequences. However, if the two types of complex had different spectral properties and if the antibiotic were to dissociate from one site (the less preferred) much faster than the other, then a two-exponential process would result. The proposed reaction scheme would be



Such a scheme results in a reaction profile characterized by two exponentials with time constants τ_1 and τ_2 related to the individual rate constants such that (see Matsen & Franklin, 1950)

$$1/\tau_1 + 1/\tau_2 = k_1 + k_2 + k_{-2}$$

$$(1/\tau_1)(1/\tau_2) = k_1 k_{-2}$$

Since k_1 and k_2 are both bimolecular rate constants, a plot of $(1/\tau_1)(1/\tau_2)$ against DNA concentration should have slope

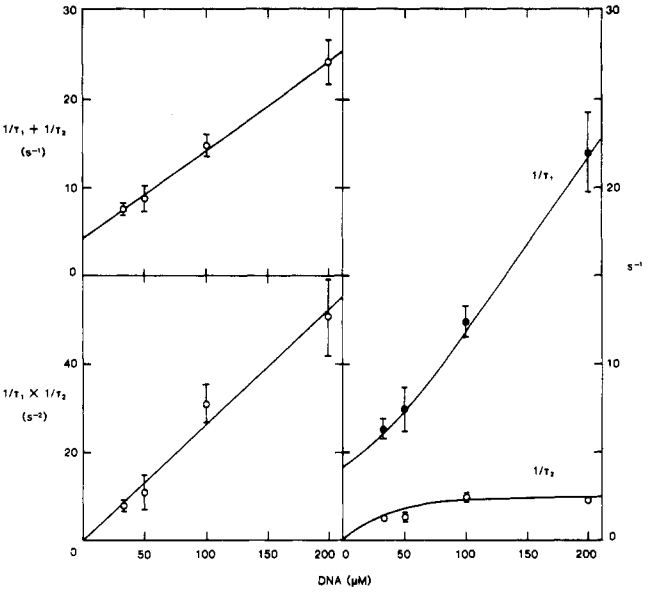


FIGURE 4: Variation of the two reciprocal time constants obtained for the interaction of echinomycin with poly(dG-dC) as a function of total nucleotide concentration. The left panel displays the sums (top) and products (bottom) of the two reciprocal time constants. The least-squares lines fitted to the points have the following parameters: $1/\tau_1 + 1/\tau_2$, slope = $(1.01 \pm 0.05) \times 10^5$ M^{-1} s^{-1}, intercept = 4.1 ± 0.5 s^{-1}; $(1/\tau_1)(1/\tau_2)$, the line is constrained to pass through the origin with slope = $(2.6 \pm 0.3) \times 10^5$ M^{-1} s^{-2}. The right panel displays the values for the actual reciprocal time constants $1/\tau_1$ and $1/\tau_2$ as a function of poly(dG-dC) concentration. The curves fitted to the points are derived from the three rate constants listed in Table II, obtained from the parameters of the plots in the left panel.

Table II: Kinetic and Thermodynamic Parameters Calculated for the Interaction between Echinomycin and Poly(dG-dC) [a]

	E (kcal mol^{-1})	ln A	$S^\ddagger$ (eu mol^{-1})
$k_1 = (6.5 \pm 1.6) \times 10^4$ M^{-1} s^{-1} $k_2 = (3.5 \pm 1.0) \times 10^4$ M^{-1} s^{-1}	9.9 ± 1.5	27.8 ± 3.6	−4.2
$k_{-2} = 4.1 \pm 0.5$ s^{-1}	16.8 ± 6.4	28.6 ± 10.2	−2.7

[a] Rate constants refer to the two-site scheme described in the text; thermodynamic quantities were calculated from the slopes and intercepts of Arrhenius plots stated in the text.

$k_1 k_{-2}$ and pass through the origin while a plot of $1/\tau_1 + 1/\tau_2$ against DNA concentration should have a slope of $k_1 + k_2$ and an intercept of k_{-2}. Plotting the sums and products of the time constants derived from the poly(dG-dC) experiments against polynucleotide concentration yields the results seen in Figure 4 (left panels). Both functions derived from the two rate constants yield reasonably good straight lines, and the parameters calculated from them are listed in Table II. Conversely, an examination of the goodness of fit between theory and the individually measured rate constants reveals satisfactory agreement (Figure 4, right panel).

At equilibrium, most of the drug will be bound to site 1, so if we assume that the observed dissociation rate constant determined with poly(dG-dC) (Fox et al., 1981) corresponds predominantly to dissociation from this tighter site, i.e., k_{-1}, we have all the numbers required to estimate relative binding constants for the two classes of sites. Calculated as quotients of the forward and reverse rate constants in this way, we find $K = 5.2 \times 10^6$ M^{-1} for site 1 and $K = 8.0 \times 10^3$ M^{-1} for site 2—a difference of nearly a 1000-fold. Possible reasons why poly(dG-dC) should display a two-exponential reaction profile whereas poly(dA-dT) yields a single exponential are considered under Discussion.

A full analysis to determine thermodynamic parameters for

each of the three rate constants in the above scheme could only be achieved by investigating the poly(dG-dC) concentration dependence of the two time constants at a series of different temperatures. Such an analysis would require prohibitively large quantities of the polynucleotide. However, a rough estimate of the thermodynamic parameters can be obtained from measurements made at a fixed polynucleotide concentration. Arrhenius plots for the temperature dependence of τ_1 and τ_2 with 100 μM poly(dG-dC) have the following parameters: τ_1, slope = $(5.0 \pm 0.7) \times 10^3$, intercept = 19.3 ± 2.5; τ_2, slope = $(6.7 \pm 1.6) \times 10^3$, intercept = 23.6 ± 5.4. Within the range studied $1/\tau_2$ can be approximated to $(k_1k_{-2})^{1/2}/2$ so that an Arrhenius plot for τ_2 should have a slope of $(\Delta E_1 + \Delta E_2)/(2R)$ and an intercept of $(1/2)$ ln $(A_1A_{-2}$[DNA]), where ΔE_1, ΔE_2, and ΔE_{-2} are the activation energies for the three reactions and A_1, A_2, and A_{-2} are their respective preexponential factors. Similarly, $1/\tau_1$ approximately equals $k_1 + k_2$, so that if we assume that $\Delta E_1 = \Delta E_2$ and $A_1 = A_2$, then an Arrhenius plot for τ_1 will have a slope of $\Delta E_1/R$ and an intercept of ln $(2A_1$[DNA]). Thermodynamic parameters derived from such a simplified analysis of the data are presented in Table II.

Discussion

Despite the low solubility of echinomycin in aqueous solutions, we have succeeded in using stopped-flow spectrometry to measure the bimolecular association kinetics of its interaction with DNA, despite, too, the small absorbance changes accompanying the binding reaction. The rate of association is slow by comparison with simple intercalators such as ethidium and proflavin (Li & Crothers, 1969; Bresloff & Crothers, 1975; Wakelin & Waring, 1980) but falls within the same range as the bimolecular portion of the reaction between actinomycin D and DNA (Müller & Crothers, 1968; Bittman & Blau, 1975).

Comparison with Equilibrium Data. It should be possible to combine the association rate constants determined here with the dissociation rate constants previously reported (Fox et al., 1981) to estimate values for the equilibrium binding constant and compare these with the values already determined by solvent partition analysis (Wakelin & Waring, 1976). If we assume that the on-rate constants for each of the three types of sites postulated in the dissociation studies are equal and are given by the association rate constants determined in this work, then the equilibrium binding constant, K_i, for each site can be determined. The overall apparent binding constant for the DNA [represented by the intercept on the r/c axis of eq 10 of McGhee & von Hippel (1974)], which we call $K(0)$, is given by the sum of the individual binding constants, each multiplied by their frequency of occurrence in the DNA helix. From the dissociation times and their relative amplitudes previously determined (Fox et al., 1981), the binding constants listed in Table I have been calculated. It is evident that the values estimated from kinetic data are 3–6 times larger than those determined directly by solvent partition analysis. This is somewhat unusual since kinetic measurements usually underestimate the true value for the binding constant, as a result of omitting one or more of the components in the reaction mechanism. There could be several explanations for this discrepancy. First, it is possible that the binding constants determined by solvent partition analysis are consistently low. Chaires et al. (1982) found that their isotherms for the binding of daunomycin to DNA determined by solvent partition analysis were much lower than those determined by optical methods. It must therefore be questioned whether in the echinomycin–DNA system the isoamyl acetate used in the solvent partition technique interferes with the binding reaction so as to yield anomalously low values for the association constant. We are currently evaluating different techniques for measuring the equilibrium binding of these very insoluble compounds to DNA, but in studies with two congeners of echinomycin, we found no differences between binding curves determined by solvent-partition and optical methods (Fox et al., 1980a,b). It is also worth noting that hitherto equilibrium binding constants have been determined from experimental data that do not extend below an r value of 0.02. The possibility exists, although we consider it unlikely, that there are large deviations from the predicted curves at low levels of ocupancy by reason of sequence selectivity, so that the quoted values have indeed underestimated the intercept on the r/c axis [cf. the results for triostin A reported by Fox et al. (1983)]. It is also conveivable, although unlikely, that the dissociation rates measured in the presence of detergent are for some reason slowed down, resulting in an underestimation of the true off-rate constants.

A third possible explanation for the discrepancy between binding constants determined by equilibrium and kinetic methods is that the measured association rate constants are actually the arithmetic average of individual rate constants for each of the different sequences with which the antibiotic interacts. It may be that the preferred, low-frequency binding sites from which the antibiotic dissociates very slowly are characterized by slower on-rate constants than this weighted average and that this is responsible for overestimating the calculated binding constants. It is quite likely that the equilibrium distribution of the drug over its various potential binding sites is very different from that initially achieved during the association reaction. If this slow rearrangement is associated with little or no spectral change, then it will not be apparent in stopped-flow experiments.

Comparison with Actinomycin D. The relative simplicity of echinomycin–DNA association kinetics reported here differs dramatically from the complexity previously described for actinomycin–DNA binding (Müller & Crothers, 1968; Bittman & Blau, 1975). We are convinced that this difference is real since in our hands actinomycin D also displays very long reaction times subsequent to the initial bimolecular reaction (see Figure 2B). There are several possible origins for this different behavior. First, for a reaction step to be detected it must be associated with a change in the spectral properties of the drug. It is conceivable that such long-term reactions between echinomycin and DNA as occur only affect the peptide portion of the molecule and do not significantly affect the chromophores. Second, echinomycin is a bifunctional intercalator and so must a priori interact with DNA via a different reaction mechanism. Since the interaction of echinomycin with natural DNAs is described by a single exponential under all the conditions investigated, even at the highest DNA concentration when the bimolecular reaction proceeds fastest, we can say that the second intercalation step is not slow compared with the intercalation of the first chromophore. As a result of this, the overwhelming majority of the drug is bound to the DNA in a bifunctional fashion, and any transient mono-intercalated intermediate does not exist for any longer than 10 ms, a short time in comparison with the association reaction itself. We currently favor the hypothesis that the slow components in the reaction profile with actinomycin are the result of "shuffling" of the drug over its potential binding sites until the optimum binding is achieved. The shuffling reaction can only be observed if the drug has significantly different absorbance spectra when bound to

different nucleotide sequences. Since the quinoxaline chromophores of echinomycin are much smaller than the phenoxazone chromophore of actinomycin, it may well be that the spectral changes on binding of quinoxalines to DNA are less sequence dependent so that the shuffling process is not accompanied by a detectable absorbance change.

It is worth considering why no long time components were detected in the present studies despite our previous observation (Fox et al., 1981) that a slow step, accounting for about 20% of the total absorbance change with a time constant of about 35 s at 20 °C, could be measured by direct mixing. A first though is that this reaction may simply be too slow, with too small an amplitude, to be detected by stopped-flow techniques. However, we favor a different explanation. It is noticeable that the thermodynamic parameters determined for the association "rearrangement" step by Fox et al. (1981) are not dissimilar from those for the dissociation of the antibiotic from its weakest (and most abundant) class of binding sites. In the process of association between the antibiotic and DNA, and especially at the very high DNA concentrations used in the previous studies, the drug must initially interact with sequences to which it binds weakly, i.e., which have a fast dissociation rate. The observed slow rearrangement will then simply reflect the migration of the drug from these weak binding sites onto sites characterized by a much longer persistence time. Since it is believed that echinomycin binds preferentially to sites with high G+C content (Wakelin & Waring, 1976) and the hypochromicity on binding to poly(dA-dT) is less than that for binding to poly(dG-dC) and natural DNAs (unpublished observations), the slow "association" step may simply reflect the drug moving from AT-rich to GC-rich sites on the DNA helix.

Reaction with Poly(dA-dT) and Poly(dG-dC). The binding model proposed for the interaction with poly(dG-dC) involves the two sites GpC and CpG, having binding constants differing by a factor of 600, although the association rate constants differ by only a factor of 2. This need not seem surprising in view of the manifest sequence selectivity of the antibiotic and does mean that at equilibrium only one of the two sites will be significantly occupied. The model thus remains consistent with the single exponential dissociation from poly(dG-dC) observed by Fox et al. (1981). According to this view, the basis for selectivity between the two types of sequences resides largely in differences between their dissociation rate constants.

The remaining question, then, is why the interaction with poly(dA-dT), which also possesses two sequences ApT and TpA, is completely described by a single exponential. Again, we can see two possible explanations. The first and least likely suggestion is that echinomycin does not distinguish between these two sequences. The second possibility, which we favor, is that the association rates for the two sequences are very different and the antibiotic binds more tightly to the faster association site. There is a plausible physical basis for this suggestion since, according to the alternating B structure proposed for poly(dA-dT) by Klug et al. (1979), the sequence TpA should be much easier to unstack than the sequence ApT. In the model for the binding of the quinoxaline depsipeptide TANDEM to poly(dA-dT) proposed by Viswamitra et al. (1981), the chromophores are inserted between the TpA sequences. According to these ideas, if the rate-limiting step in the interaction of quinoxaline antibiotics with DNA is the unstacking of the base pairs, then the sequence that is kinetically preferred is also the one to which the antibiotic binds more tightly. The selectivity between ApT and TpA is consequently a function of both the on- and off-rate constants. The difference in association rate constants may not be apparent with poly(dG-dC) since this polymer is said to adopt a more classical B-form structure (Cohen et al., 1981).

Registry No. Echinomycin, 512-64-1; poly(dA-dT), 26966-61-0; poly(dG-dC), 36786-90-0.

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