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ARTICLE *in* BIOCHEMISTRY · OCTOBER 1981

Impact Factor: 3.02 · DOI: 10.1021/bi00523a020 · Source: PubMed

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Kinetics of the Interaction between Echinomycin and Deoxyribonucleic Acid†

Keith R. Fox, Laurence P. G. Wakelin, and Michael J. Waring*

ABSTRACT: The kinetics of dissociation of the echinomycin–deoxyribonucleic acid (DNA) complex have been studied by using the technique of detergent sequestration of the free antibiotic. With natural DNAs the decay curve was fitted by a three-exponential function whereas that from synthetic DNAs was completely described by a single exponential. The magnitude and relative proportions of each of the time constants seen with natural DNAs varied, depending upon both the binding ratio and the base composition of the DNA. This is interpreted as representing the parallel dissociation of echinomycin from different sites on the heterogeneous DNA lattice, each class of sites having a different affinity for the antibiotic. A small proportion of the total dissociation occurs very slowly and corresponds to dissociation of the antibiotic from its most preferred binding sites; these are more abundant in GC-rich DNA and appear to contain three (or four) GC nucleotide pairs. The dissociation rate constants also varied with the total DNA concentration. This is explained by invoking a bimolecular dissociation pathway termed "facilitated dissociation". The speeding up of rate constants with an increasing level of antibiotic binding may indicate the occurrence of cooperativity and/or long-lived, progressive changes in the DNA structure as more echinomycin is bound. A proportion of the association reaction occurs sufficiently slowly to be measurable by direct spectrophotometry; it is practically independent of DNA concentration and probably corresponds to an intramolecular rearrangement of the DNA-bound antibiotic in the complex.

Quinoxaline antibiotics are produced by several species of streptomycetes and 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 deoxyribonucleic acid (DNA)[1] (Ward et al., 1965; Sato et al., 1967; Waring & Makoff, 1974; Waring, 1979). The best known member of the group is echinomycin (quinomycin A) whose structure, shown in Figure 1, has been revised from that originally proposed (Dell et al., 1975; Martin et al., 1975). It was the first reported example of a bifunctional DNA intercalating agent (Waring & Wakelin, 1974).

Wakelin & Waring (1976) showed that echinomycin yields average binding constants for natural DNAs of differing base composition which may vary by an order of magnitude. While there is no direct correlation between the measured binding constants and the gross base composition of the DNA, the antibiotic tends to bind more tightly to DNAs rich in G + C residues. It has been suggested, however, that the most preferred binding site may contain all four bases, since the highest binding constant observed was with *Micrococcus lysodeikticus* DNA and not with poly(dG-dC) (Wakelin & Waring, 1976; Waring, 1979). One purpose of the present study was to investigate further the nature of sequence preferences in the binding of echinomycin to DNA.

The quinoxaline group of antibiotics can be usefully compared with the actinomycins for which the kinetics of DNA binding were first described by Müller & Crothers (1968). They showed that the dissociation reaction occurred by a slow, multistep process and that its complete description required three exponential terms. Müller & Crothers (1968) attributed this complex kinetic behavior to sequential steps involving conformational changes in the peptide backbone of the antibiotic, accompanying the strong interaction between the antibiotic molecule and the DNA helix. More recently, Krugh et al. (1979) have attributed the occurrence of three components in the dissociation of actinomycin D from calf thymus DNA to the existence of three different intercalative binding sites on the helix. They obtained a single-exponential dissociation curve from poly(dG-dC), supporting the contention that the multiexponential decay obtained with calf thymus DNA was due to site heterogeneity.

In this paper we describe experiments on the nature and complexity of the echinomycin–DNA dissociation reaction which support the concept of site heterogeneity attributable to sequence preferences in the binding of this antibiotic to DNA. The slowest dissociation reaction, ascribed to the most preferred binding sites and those most likely to be relevant to the action of the antibiotic in vivo, has been characterized in detail. A novel mechanism involving bimolecular dissociation is also invoked to explain the variation of dissociation rate constants with total DNA concentration.

Materials and Methods

Chemicals. Except where otherwise stated, all experiments were conducted in Hepes–NaOH buffer, pH 7.0, ionic strength 0.01, designated 0.01 SHE. A stock solution containing 0.2 M Hepes, 1 mM EDTA, and 0.94 M NaCl was adjusted to pH 7.0. 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., Basle, Switzerland. Calf thymus DNA (highly polymerized sodium salt, type I) and *Escherichia coli* B DNA (type VIII) were purchased from Sigma Chemical Co. DNA from bacteriophage T2 was prepared as previously described (Waring, 1965). Ribosomal RNA was extracted from the ribosomes of *E. coli* by two

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[1] Abbreviations used: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

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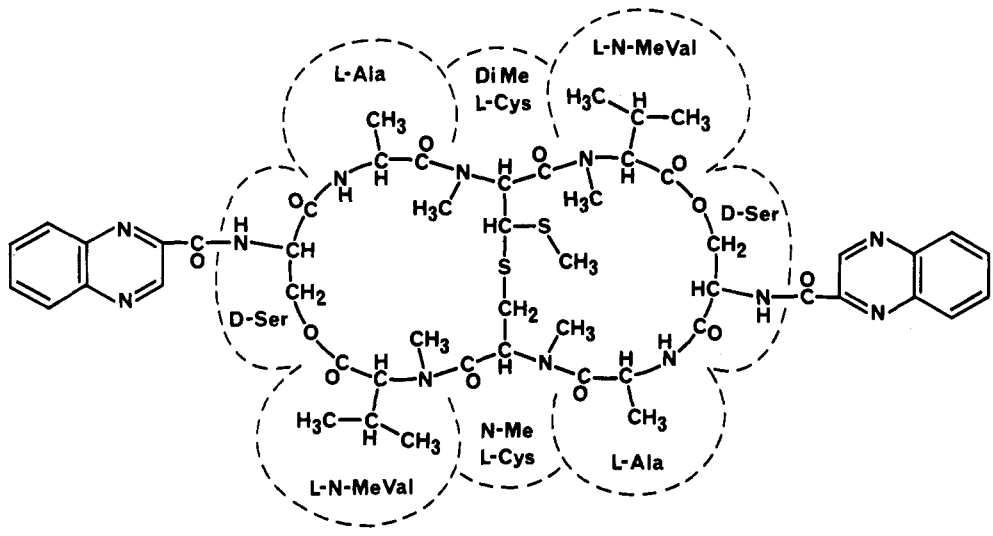

FIGURE 1: Structure of echinomycin.

successive treatments with phenol followed by ethanol precipitation. For *M. lysodeikticus* and *Clostridium perfringens* DNAs 5-g quantities of lyophilized cells were purchased from Sigma Chemical Co., and the DNA was extracted essentially by the method of Marmur (1961). Poly(dA-dT) was obtained from the Boehringer Corp.; poly(dG-dC) was from P-L Biochemicals, Milwaukee, WI. Poly(rA)·poly(rU) was prepared by mixing equimolar solutions of poly(rA) and poly(rU) (Sigma). All synthetic polymers were found to be readily soluble in 0.01 SHE and were used as supplied without further purification. Nucleic acid concentrations are expressed with respect to nucleotides, on the basis of an assumed value of $\epsilon_{p,260}$ of 6600 except for *M. lysodeikticus* DNA (6300) (Tubbs et al., 1964). Concentrations of synthetic DNAs were based on the values of ϵ_p given by Wells & Wartell (1974).

Kinetics of Dissociation of Echinomycin–DNA Complexes. (*1*) *Experimental Procedures.* All complexes were prepared by a "solid shake" procedure similar to that used by Lee & Waring (1978a). A small amount of solid antibiotic was shaken vigorously with a DNA solution, followed by clarification by filtration and/or centrifugation. The molar ratio of total drug to DNA nucleotides (D/P) was then measured by dissociating a portion with an equal volume of dimethyl sulfoxide (Me_2SO) and measuring the absorbance at 325 nm, against an appropriate optical reference. The extinction coefficients of echinomycin were as tabulated in Waring (1979).

All dissociation experiments were performed in 40-mm optical path length cuvettes, except those at DNA concentrations of 1 mM and some of those with synthetic DNAs for which 10-mm cells could be used. Typically, 3.2 mL of an antibiotic–DNA complex was mixed with 0.8 mL of a solution of sodium dodecyl sulfate $NaDodSO_4$ (10% w/v) buffered to pH 7.0 in 0.01 SHE. The solution was rapidly mixed by inverting the cuvette several times, taking care to avoid trapping of air bubbles in the solution. The cuvette was then placed in the spectrophotometer and the recording device started. The fastest that any reading could be taken was ~10 s after the addition of the $NaDodSO_4$, although more typically 15 s had elapsed. Therefore, any portion of the total decay occurring with a time constant of 4 s or less would not be observable, since it would be largely complete by the start of recording.

Early experiments were performed with a Unicam SP500 Series II spectrophotometer coupled to a Kipp & Zonen chart recorder. Readings of transmittance were transferred onto computer by hand with a sampling frequency dependent on the rate of decay; at least 100 points were collected per decay. Readings were continued for at least 4 times the longest observed dissociation decay constant. Subsequently, this system was replaced by a Unicam SP8 200 spectrophotometer coupled to its own chart recorder and a paper tape punch. Readings were taken in the absorbance mode and punched at four points per second. After the first 100 s the remainder of the decay was read from the chart recording and fed manually into the computer. Both systems gave essentially the same results, but with greater accuracy from the latter system, due largely to the increased number of data points available for analysis. Because of the errors inherent in decomposing a multiexponential decay, each experiment was repeated up to 5 times.

(*2*) *Analysis of Kinetic Data.* The decay was analyzed by using 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 370/165 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

$$A_t = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} + A_3 e^{-t/\tau_3}$$

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 expressed as a percentage of the total absorbance change. The program provides several statistical parameters for checking that the data have been properly handled and tests for overdetermination. Its characteristics have been described fully by Johnson & Schuster (1974) and Wakelin & Waring (1980). Tests with synthetic data of known parameters, perturbed with appropriate levels of random noise, showed that under the conditions employed the program was capable of adequately resolving the three exponentials [for details see Johnson & Schuster (1974) and Wakelin & Waring (1980)].

Detergents and Conditions Used. Preliminary experiments were undertaken to establish that the technique of dissociation by addition of detergent yields a reliable measure of the true dissociation rate constant(s). The use of $NaDodSO_4$ to disrupt drug–DNA complexes in this manner has been described by several authors (Müller & Crothers, 1968; Krugh et al., 1979; Behr et al., 1969; Grant & Philips, 1979; Shafer et al., 1980; Wilson et al., 1976). In agreement with the conclusions of

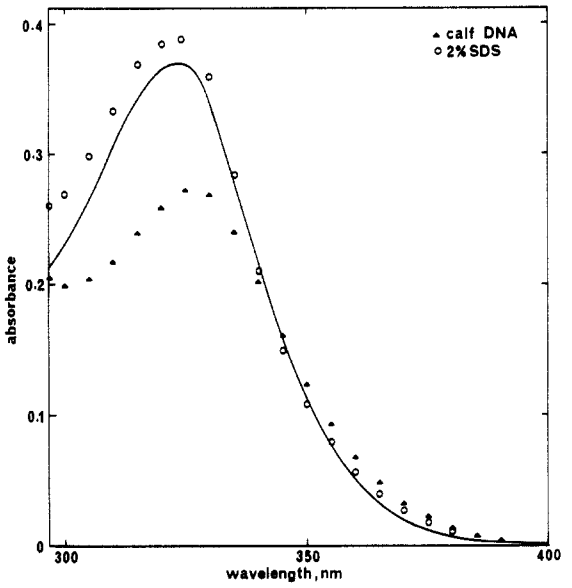

FIGURE 2: Effect of calf thymus DNA and $NaDodSO_4$ on the absorption spectrum of echinomycin. The continuous line represents the spectrum of 3.2 μM echinomycin in 0.01 SHE buffer measured in 100-mm light path quartz cuvettes; (▲) 3.2 μM echinomycin in the presence of 500 μM calf thymus DNA; (○) 3.2 μM echinomycin in the presence of 500 μM calf thymus DNA and 2% $NaDodSO_4$ (w/v) after equilibration to allow complete dissociation.

these workers, we were unable to detect any changes in the secondary structure of DNA attributable to the presence of detergent, assessed by a variety of optical and hydrodynamic methods. This procedure rests on the assumption that the drug dissociates from the DNA in a normal fashion and is merely sequestered by the detergent before it can reassociate with the helix. In the case of anthracycline antibiotics, at least, it is certain that the process of sequestration must occur fast compared with the initial process of dissociation (Wilson et al., 1976). The absorption spectrum of echinomycin in the presence of 500 μM DNA before and after addition of 2% $NaDodSO_4$ (w/v) is shown in Figure 2. The antibiotic spectrum displays typical hypochromic and bathochromic shifts on binding to DNA [see Waring et al. (1975)], and this effect is reversed on adding $NaDodSO_4$. There is even a slight hyperchromism in the presence of the detergent, a factor which improves the magnitude of the total observed absorbance changes in dissociation experiments. The spectral difference is greatest at 320 nm, and therefore this wavelength was chosen for measuring the kinetics of dissociation.

The capacity to promote dissociation of echinomycin–DNA complexes is not common to all detergents. Triton X-100, Triton X-405, sodium deoxycholate, *N*-lauroyl sarcosinate, and Nonidet P-42 were all tested at 2% (w/v) final concentration but failed to produce time-dependent spectral changes; presumably, these detergents are unable to sequester free antibiotic molecules. In separate experiments it was established that the optimum concentration of $NaDodSO_4$ lies close to 2% (w/v). The correct choice of detergent concentration is very important since sufficient detergent must be present to sequester all the dissociated drug molecules. However, the more $NaDodSO_4$ that is added, the higher the resulting ionic strength (2% $NaDodSO_4$ added to a buffer of ionic strength 0.01 yields a total ionic strength of 0.093), and solubility problems can be encountered, especially at low temperatures. A series of echinomycin–calf thymus DNA complexes were treated with $NaDodSO_4$ at final concentrations ranging from 0.1 to 8.0% (w/v). It was found that τ_1 appeared to be unaffected by the $NaDodSO_4$ concentration over the entire range. The same applied to τ_2, except that it was not detectable below 0.5% $NaDodSO_4$. With 0.1% $NaDodSO_4$ only ~30% of the expected total amplitude was observed, presumably because a large proportion of the drug molecules represented by τ_2 and τ_3 still remained bound to the DNA. The variation of τ_3 with $NaDodSO_4$ concentration was insignificant above 2% but became pronounced at lower detergent concentrations, indicating apparently faster release. This is probably because at the lower $NaDodSO_4$ concentrations there were not sufficient detergent molecules present to sequester all the drug; as a result the measured rates would not be true dissociation rates but would represent an approach to a new equilibrium with some of the drug sequestered by the $NaDodSO_4$ and some still bound to the DNA. Accordingly, the final detergent concentration employed in all subsequent experiments was 2% (w/v). In addition, a check was kept on the total observed amplitude to ensure that all the drug (within experimental error) had dissociated. The pH of the dissociated complexes was also monitored to ensure that they had not been subjected to a pH jump in addition to the changes in $NaDodSO_4$ concentration. In all cases the final pH was found to be close to 7.0.

Kinetics of Association between Echinomycin and DNA. The binding reaction was initiated by shaking 3.0 mL of a nearly saturated solution of echinomycin with 1.0 mL of a concentrated DNA solution in 40-mm optical cuvettes. The hypochromic absorbance change was then followed at 320 nm in a Unicam SP8 200 spectrophotometer. Data were recorded on paper tape at four points per second in a manner analogous to that described for dissociation experiments. The temperature was maintained at 12.5 °C by means of a thermostated 40-mm cell holder through which water was pumped from a Haake water pump with the heater opposing the action of a cooling coil. For most experiments very high DNA concentrations were used. Under these conditions the total free DNA concentration remains practically constant throughout the experiment and the association process becomes a pseudo-first-order reaction. With 5 μM echinomycin and a total DNA concentration of 1 mM, the final value or r (antibiotic molecules bound per DNA nucleotide) is 0.005. From eq 9 of McGhee & von Hippel (1974) the effective free DNA concentration present at equilibrium is calculated to be 925 μM (by using a value for the site size $n = 8$). Thus, even for this 200-fold excess of DNA over drug concentration there is still a 7% change in the effective DNA concentration during the course of the reaction. The observed decay was fitted by using the same program as for the analysis of dissociation kinetics, by decomposing the decay into as many exponentials as was necessary.

Results

Representative curves for the dissociation of echinomycin from poly(dG-dC) and poly(dA-dT) are shown in Figure 3a. The curves are displayed in the usual manner for an exponential decay, i.e., with the ordinate as the natural logarithm of the fractional absorbance change and the abscissa representing time. It is evident from the linear nature of these plots, extending over several half-lives, that the time dependence of the decay is adequately described by a single exponential. The values for the time constants, averaged over several observations, were 25.0 ± 0.8 s for poly(dA-dT) and 80.6 ± 1.9 s for poly(dG-dC). Thus although the dissociation from both DNAs is adequately described by a single exponential, the time constant for the decay is different for the two polynucleotides and therefore appears to depend on the nucleotide composition. The cost of these synthetic DNAs precluded the possibility

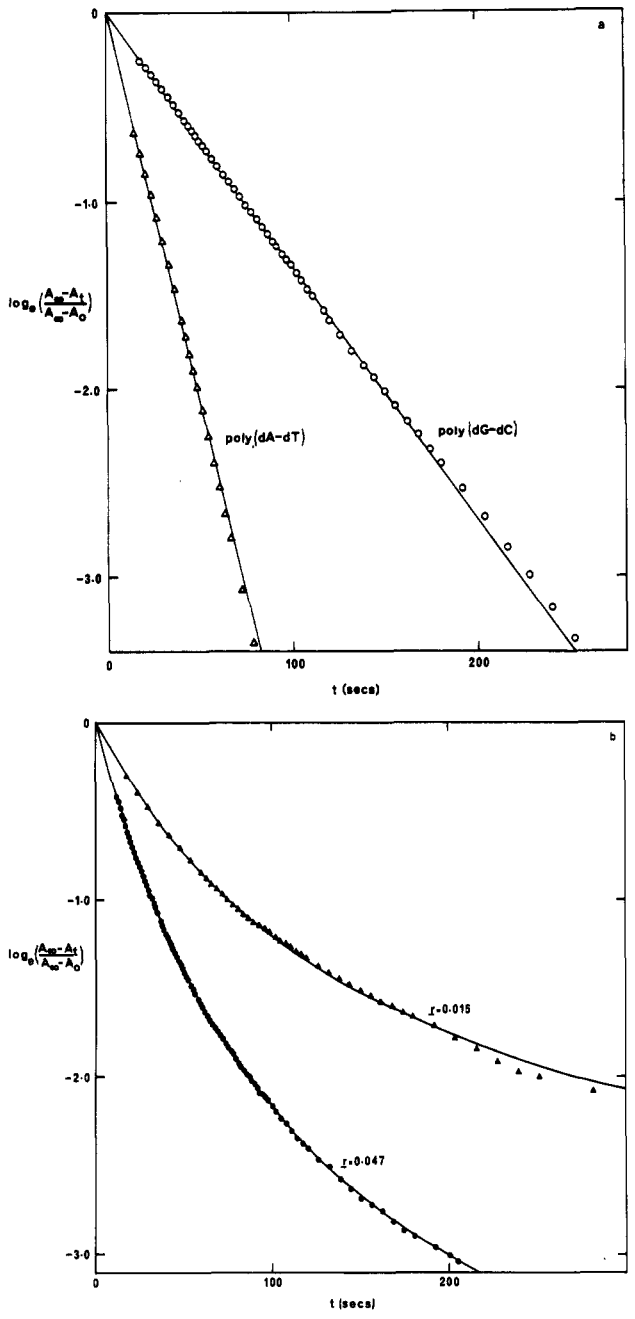

FIGURE 3: Dissociation of echinomycin from synthetic and natural DNAs. The ordinate represents the natural logarithm of the fractional absorbance change. All complexes were prepared with 500 μM DNA. (Panel a) (○) Poly(dG-dC), D/P = 0.05; (△) poly(dA-dT), D/P = 0.05. (Panel b) Calf thymus DNA: (●) D/P = 0.047; (▲) D/P = 0.015. The lines fitted to the data in panel a are single-exponential fits characterized by time constants of 24.3 s for poly(dA-dT) and 74.1 s for poly(dG-dC). Those drawn in panel b are three-exponential decay curves, fitted as described in the text and characterized by the following parameters: for r = 0.014, τ_1 = 32.2, τ_2 = 79.1, and τ_3 = 585 s and A_1 = 40%, A_2 = 41%, and A_3 = 19%; for r = 0.047, τ_1 = 18.4, τ_2 = 53.1, and τ_3 = 363 s and A_1 = 55%, A_2 = 38%, and A_3 = 7%. In each case data were collected for at least four times the longest time constant.

of a detailed examination of their dissociation properties. However, it was possible to show that the dissociation rate constant from poly(dA-dT) was largely independent of the binding ratio of the complex over the range of r from 0.032 to 0.096.

Dissociation curves for calf thymus DNA are illustrated in Figure 3b. The curves shown correspond to input ratios (D/P, molar ratio of drug molecules to DNA nucleotides) of 0.015 and 0.047. In both cases the DNA concentration was 500 μM.

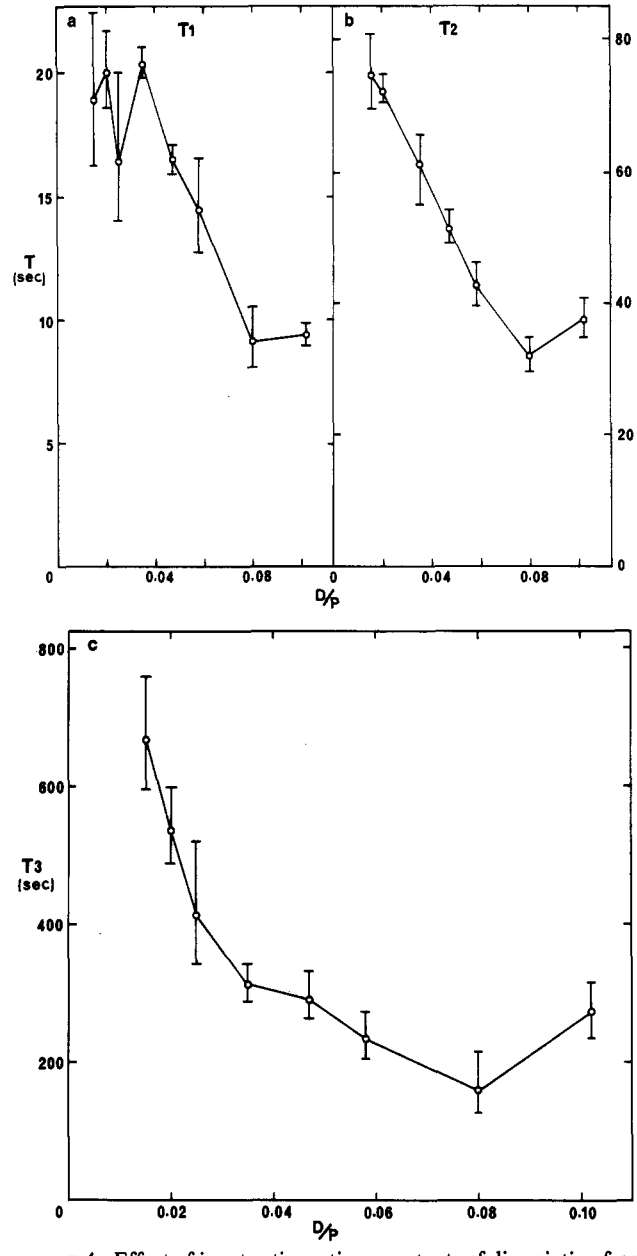

FIGURE 4: Effect of input ratio on time constants of dissociation from calf thymus DNA. The abscissa represents the molar ratio (D/P) of total echinomycin molecules to DNA nucleotides. Time constants were derived from an analysis of the data according to the program of Johnson & Schuster (1974) as described in the text; error bars represent the standard error from five determinations. (a) τ_1; (b) τ_2; (c) τ_3.

It is possible to discern two important characteristics of the decay from these curves. First, the decay is not represented by a straight line and therefore must be analyzed in terms of a multiexponential function. Second, the shape of the curve is different at the two input ratios, suggesting that the parameters of decay must be dependent upon the level of binding.

Variation of Decay Parameters with Initial (Input) Ratio. In this series of experiments the total DNA concentration was maintained at 500 μM while D/P was varied over the whole accessible range. The dissociation curves were analyzed in terms of a three-exponential decay as described under Materials and Methods. The resulting time constants and their relative amplitudes are shown in Figures 4 and 5. It can be seen that each of the three components of the decays becomes slower at lower input ratios, the effect being most pronounced for τ_3. At each input ratio three components were readily resolved, with τ_1 and τ_2 each contributing 40–50% of the total

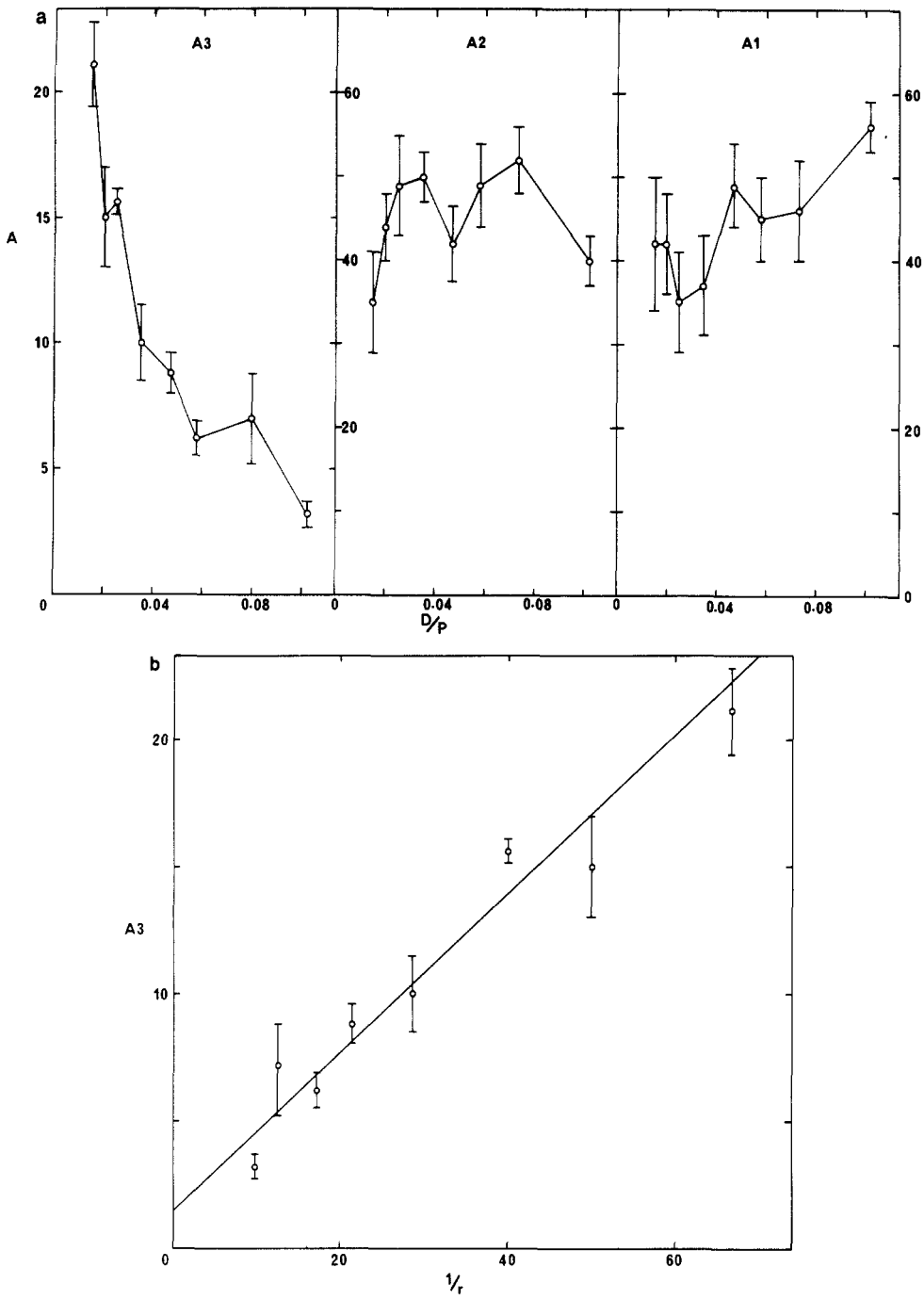

FIGURE 5: Effect of input ratio on relative amplitudes of time constants for dissociation from calf thymus DNA. In (a) the relative amplitudes of the time constants are expressed as a percentage of the total absorbance change. Conditions were as described in Figure 4. In (b) the data for A_3 are replotted as a function of the reciprocal of the input ratio (i.e., the molar ratio of DNA nucleotides to antibiotic molecules). The line drawn through the points represents a least-squares fit; its slope is 0.0031 ± 0.0003 and its intercept 1.5 ± 1.7.

amplitude and the remainder accounted for in τ_3. The relative amplitudes were found to vary with input ratio as illustrated in Figure 5a. Although the changes in A_1 and A_2 reveal little or no systematic variation, there is a definite trend in the variation of A_3 with input ratio. At $r = 0.015$ this component represented ~22% of the total observed absorbance change whereas at $r = 0.1$ it was only responsible for 2–3% of the total decay. This behavior can be accounted for by postulating that the complexity of the dissociation is due to the existence of at least three classes of intercalation sites on the DNA. If each type of site has a different microscopic binding constant for the antibiotic, then the fraction of ligand molecules bound by each class of binding site will be expected to vary with the total binding ratio. At very low input ratios the drug should bind preferentially to the sites with the largest binding constant. At higher input ratios these sites will become saturated and the drug will bind to the weaker sites. If the dissociation from each class of binding sites can be represented by a single exponential, then the relative contribution of each time constant to the total dissociation should vary as a function of the relative population of the sites. This can be put on a more quantitative basis by assuming that the largest time constant corresponds to the existence of a small number of tightly binding sites on the DNA, which are always fully occupied over the entire range of input ratios studied. If these sites become fully saturated at an r value of x, then, at any binding ratio (r) greater than this, the fraction of the total drug molecules bound to these sites will be x/r. According to this model, plotting A_3 against $1/r$ should yield a straight line with gradient x. Such a graph of A_3 against $1/r$ is shown in Figure

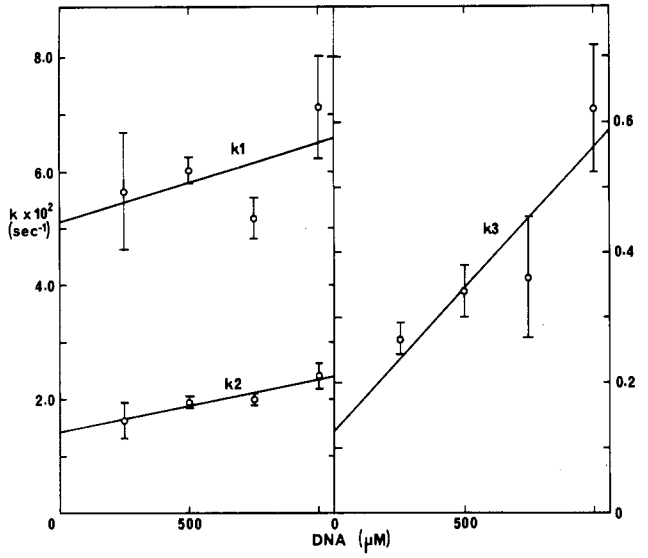

FIGURE 6: Dependence of dissociation rate constants on DNA concentration. The ordinate represents the dissociation rate constant ($k = 1/\tau$), and the abscissa represents the total DNA concentration in the complex. For each determination the binding ratio r was maintained close to 0.05 drug molecules per nucleotide. The lines drawn through the points represent least-squares fits to the experimental data and are characterized by the following parameters: k_1, slope = 14 ± 15 M^{-1} s^{-1}, intercept = 0.051 ± 0.012 s^{-1}; k_2, slope = 9.3 ± 2.0 M^{-1} s^{-1}, intercept = 0.014 ± 0.002 s^{-1}; k_3, slope = 4.3 ± 1.4 M^{-1} s^{-1}, intercept = 0.0013 ± 0.001 s^{-1}.

5b. The line drawn was fitted by the method of least squares: its slope is 0.0031 ± 0.0003. The reasonable fit to the experimental points provides support for our interpretation that the longest time constant represents the dissociation of the drug from a small number of tightly binding sites on the DNA. These must be saturated at an r value of 0.0031, representing about three binding sites per thousand nucleotides. Although this simple analysis must necessarily be considered an approximation to the real situation, it may be expected to yield a lower limit to the frequency of high-affinity sites, since it assumes that they are always fully occupied. This will only be strictly true if echinomycin binds to the A_3 sites much more tightly than to the other sites. If this is not the case, even at very low input ratios the large number of low-affinity binding sites may effectively compete with the high-affinity sites for binding of the antibiotic. However, all the dissociation experiments were performed with starting ratios at least 5 times the apparent frequency of high-affinity sites, so the estimate of their frequency, though only approximate, should not be seriously in error.

Variation with Absolute DNA Concentration. For a truly monomolecular reaction, such as might be expected for a dissociation reaction, the rate constant should be independent of the starting concentration of the reactant. In this case, altering the absolute concentration of the starting complex should have no effect on the decay constants for the system. Accordingly, the dissociation time was measured as a function of DNA concentration, the other parameters, such as the input ratio of drug to nucleotides, being held constant. Contrary to expectation, the dissociation rate constants were found to vary with the total DNA concentration at a fixed value of r (Figure 6). The changes in k_1 and k_2 were relatively small, possibly insignificant, but k_3 changed by a factor of 3 over the 4-fold range of DNA concentration. This surprising observation was repeated several times with 500 μM and 1 mM DNA so as to rule out other possible explanations, such as changes in temperature. Thus, the dissociation cannot be considered merely as a straightforward first-order decay process. A model, derived from the ligand-transfer ideas of Bresloff & Crothers (1975), in which the dissociation consists of two simultaneous reactions, may be proposed to account for this observation. The first reaction is simply the monomolecular dissociation of the drug from DNA to a free state in solution. The second pathway involves a bimolecular reaction in which the drug is "helped" off the DNA by interaction with another nucleic acid molecule. At low DNA concentrations the first mechanism will predominate, and the observed rate will be close to that of the monomolecular reaction alone. However, with higher DNA concentrations the bimolecular pathway for dissociation will become more important and so speed up the overall dissociation rate. If this interpretation is correct, then plotting the rate constant ($k = 1/\tau$) against DNA concentration should give a straight line with the intercept on the ordinate equal to the monomolecular rate constant, the gradient representing the bimolecular rate constant. This is the form in which the data are presented in Figure 6. The abscissa was chosen to represent the total DNA concentration rather than the free DNA concentration at equilibrium given by eq 9 of McGhee & von Hippel (1974), since there is no a priori reason why the second, bimolecular reaction pathway should require the same site size on the DNA as the usual intercalation process. From this plot for k_3 the monomolecular rate constant is calculated to be 1.27×10^{-3} s^{-1} and the bimolecular constant 4.3 M^{-1} s^{-1}. It is possible that the apparent bimolecular reaction of a complexed molecule with another DNA molecule occurs via some nonspecific association between the "back" of the peptide portion of the antibiotic and a second uncomplexed DNA molecule as originally envisaged for actinomycin by Müller & Crothers (1968) and later considered to account for "direct ligand transfer" of bound ethidium (Bresloff & Crothers, 1975). If so, the process might be expected to occur with all nucleic acids, even those with which the quinoxaline antibiotics show no strong, specific interaction. This hypothesis was tested by observing the effect of adding RNA to a complex of echinomycin with DNA. A complex having $r = 0.05$ was prepared with 500 μM calf thymus DNA, and to this was added a small volume of concentrated *E. coli* ribosomal RNA to give a final RNA concentration of 500 μM, so that the total nucleic acid concentration was 1 mM. Previous binding studies (Wakelin & Waring, 1976) have shown that echinomycin shows little or no interaction with RNA so that in this mixed complex essentially all of the drug should still be bound to the DNA. The dissociation properties of this complex were then measured and, on analysis, yielded the following time constants: τ_1 = 9.9, τ_2 = 41, and τ_3 = 152 s. These values are very similar to the rates observed with 1 mM DNA alone (τ_1 = 14.0, τ_2 = 42, and τ_3 = 161 s); thus, the experiment tends to support this unusual mechanism involving facilitated dissociation of the drug via interaction with another nucleic acid.

Effect of DNA Base Composition on Parameters of Dissociation. If the complicated character of the dissociation from natural DNAs is a result of nonequivalence in potential binding sites and if each resolved exponential represents dissociation from a discrete class of site, then the relative proportions (i.e., amplitudes) of the time constants at fixed r and DNA concentration should vary as a function of the base composition of the DNA. Table I records the dissociation times and amplitudes for echinomycin measured with a variety of natural DNAs of differing base composition, together with the results for synthetic DNAs. Each of these experiments was performed at the same DNA concentration and antibiotic binding ratio.

Table I: Parameters of Dissociation for Various DNAs[a]

DNA	% G + C	τ_1 (s)	τ_2 (s)	τ_3 (s)	A_1	A_2	A_3	$K_{(0)}$ (μM^{-1})
C. perfringens	30	17.1 ± 2.5	58.3 ± 3.4		56 ± 5	44 ± 5		0.34
bacteriophage T2	34	22.0 ± 0.7	77.0 ± 2.0	296 ± 21	35 ± 1.5	42 ± 1	22 ± 1	0.78
calf thymus	42	16.5 ± 0.6	51.0 ± 2.0	297 ± 35	49 ± 5	42 ± 4.5	9 ± 1	0.55
E. coli	50	12.7 ± 3.5	51.0 ± 5.5	216 ± 7	25 ± 3.5	43 ± 3	32 ± 2	0.98
M. lysodeikticus	72		42.5 ± 2.0	216 ± 7		27 ± 2	63 ± 2	3.10
poly(dG-dC)	100	80.6 ± 1.9						0.55
poly(dA-dT)	0	25.0 ± 2.8						0.31

[a] The time constants τ_1, τ_2, and τ_3 and their respective relative amplitudes A_1, A_2, and A_3 (expressed as percent of total absorbance change) were determined from observed decays as described in the text. All refer to an r value of 0.05 at 500 μM DNA at room temperature. Values for $K_{(0)}$, the equilibrium binding constants, are taken from Wakelin & Waring (1976).

It can be seen that the actual magnitudes of the time constants show little variation with base composition between the natural DNAs. However, the relative contribution of each component to the total decay varies considerably. For *C. perfringens* and *M. lysodeikticus* DNAs the dissociation curves were completely described by two exponentials alone. For *C. perfringens* DNA these two time constants correspond very well with τ_1 and τ_2 observed with calf thymus DNA, whereas with *M. lysodeikticus* DNA the time constants are clearly similar to τ_2 and τ_3 of calf thymus DNA. It therefore appears that the fast dissociating class of sites is not present in significant amounts with *M. lysodeikticus* DNA, whereas the slowly dissociating sites are below the limit of detection in *C. perfringens* DNA. For both bacteriophage T2 and *E. coli* DNAs the decay was described by the sum of three exponentials with approximately the same time constants as those of calf thymus DNA but different relative amplitudes. There appears to be a good correlation between the contribution of τ_3 to the total decay (i.e., the relative magnitude of A_3) and the measured equilibrium binding constants for the various DNAs. This is especially noteworthy for bacteriophage T2 DNA. This DNA has its wide groove substantially occluded by sugar substituents on the 5-(hydroxymethyl)cytosine residues so that it should not be able to accommodate large ligands such as quinoxaline antibiotics in its major groove. On the basis of binding studies with this DNA, it was suggested that quinoxalines bind in the narrow groove (Waring & Wakelin, 1974). The equilibrium binding constant found for this DNA was in fact somewhat larger than might have been expected on the basis of its gross G + C content: T2 DNA has only 35% G + C residues yet it binds echinomycin better than does calf thymus DNA (42% G + C). The observed amplitude of τ_3 for T2 DNA is also consistent with this unexpectedly large binding constant.

An attempt was made to measure the dissociation of echinomycin from poly(rA)·poly(rU), since Wakelin & Waring (1976) reported that it displays weak interaction with this polymer. However, the highest input ratio that could be attained was 0.02, and on addition of $NaDodSO_4$ to this complex the dissociation was complete before any readings could be taken. The dissociation from this polyribonucleotide is therefore much faster than that seen with DNAs, suggesting that the complex formed must be quite different.

The effect of varying input ratio on the dissociation times and amplitudes for *E. coli* DNA is shown in Table II. The trends are similar to those seen with calf thymus DNA, yet with a greater proportion of the total amplitude in the slow dissociation. The appearance of three dissociation time constants and their variation with r therefore seem to be features of most DNAs and are not peculiar to calf thymus DNA.

Thermodynamics of Decay. The effect of temperature on the three dissociation times is shown in Figure 7a for an r value of 0.050 and in Figure 7b for an r value of 0.015. The results are presented in the form of Arrhenius plots of ln k against

Table II: Effect of Input Ratio on Parameters of Dissociation from *E. coli* DNA[a]

D/P	τ_1 (s)	τ_2 (s)	τ_3 (s)	A_1	A_2	A_3
0.026	20 ± 2	69 ± 3	348 ± 6	14 ± 3	38 ± 2	48 ± 2
0.049	12.7 ± 3.5	51 ± 5.5	216 ± 7	25 ± 3.5	43 ± 3	32 ± 2
0.087	6.3 ± 0.9	29.6 ± 2.0	128 ± 10	35 ± 4	40 ± 3	24 ± 5

[a] Time constants τ_1, τ_2, and τ_3 and their respective relative amplitudes A_1, A_2, and A_3 were estimated from the observed decays as described in the text. All values refer to 500 μM *E. coli* DNA at room temperature.

Table III: Energy of Activation for the Dissociation of Echinomycin from Calf Thymus DNA[a]

		ΔE (kJ mol^{-1})	A (s^{-1})	$\Delta S^{\ddagger}$ (eu mol^{-1})
$r = 0.050$	τ_1	14.1 ± 2.2	1.69×10^9	−17.4
	τ_2	18.3 ± 1.0	4.95×10^{11}	−6.0
	τ_3	24.9 ± 2.0	6.83×10^{15}	+13.1
$r = 0.015$	τ_2	17.4 ± 1.3	9.7×10^{10}	−9.3
	τ_3	20.0 ± 1.9	9.8×10^{11}	−4.6

[a] Thermodynamic parameters were determined from the Arrhenius plots shown in Figure 7 as described in the text. All refer to 500 μM calf thymus DNA at room temperature, with a starting ionic strength of 0.01 to which $NaDodSO_4$ was added to give a final concentration of 2% (w/v).

the inverse of the absolute temperature. At all temperatures the relative amplitudes associated with each time constant were found to be the same as at room temperature, within experimental error. The slope of the Arrhenius plot yields $-\Delta E/R$, where ΔE is the activation energy of the process and R is the gas constant. The intercept on the ordinate gives ln A, where A is the preexponential factor in the equation $k = Ae^{-\Delta E/(RT)}$. The value of A gives an indication of steric and entropic factors involved in the activation process. The entropy of activation, $\Delta S^{\ddagger}$, can be derived from the value of A by assuming that, for a unimolecular reaction, $A = (kT/h)e^{\Delta S^{\ddagger}/R}$, where k is Boltzmann's constant and h is Planck's constant (Frost & Pearson, 1961). If the "normal" value of kT/h is taken as 10^{13} s^{-1}, then $e^{\Delta S^{\ddagger}/R}$ is a factor determining whether the reaction goes slower or faster than normal. If $\Delta S^{\ddagger}$ is positive, it corresponds to a more probable activated complex, and the reaction proceeds faster than normal. If $\Delta S^{\ddagger}$ is negative, then the activated complex becomes less probable and the rate correspondingly slower. The values of ΔE, A, and $\Delta S^{\ddagger}$ for each time constant at the two different r values are presented in Table III. At higher temperatures it was often not possible to resolve the decay into three exponentials, and the total observed amplitude was much reduced. This was taken to signify that τ_1 had become too fast to measure, and therefore the resolvable exponentials were termed τ_2 and τ_3. At still higher temperatures τ_2 vanished as well, and the decay was adequately fitted by a single exponential assumed to represent

Table IV: Effect of Ionic Strength on Parameters of Dissociation[a]

ionic strength	τ_1 (s)	τ_2 (s)	τ_3 (s)	A_1	A_2	A_3	$K_{(0)}$ (μM^{-1})
0.01	16.5 ± 0.6	51.0 ± 2.0	297 ± 35	49 ± 5	42 ± 4.5	9 ± 1	0.548
0.1	5.8 ± 0.7	19.4 ± 0.8	155 ± 10	47 ± 3	47 ± 3	6 ± 1	0.147
0.5		13.7 ± 0.9	140 ± 7		89 ± 0.9	11 ± 0.9	0.104

[a] The time constants τ_1, τ_2, and τ_3 and their respective relative amplitudes A_1, A_2, and A_3 were determined as described in the text. The ionic strength refers to the initial conditions before addition of $NaDodSO_4$. All values are for 500 μM calf thymus DNA and $r = 0.050$ at room temperature. Values for $K_{(0)}$, the equilibrium binding constants, are taken from Wakelin & Waring (1976).

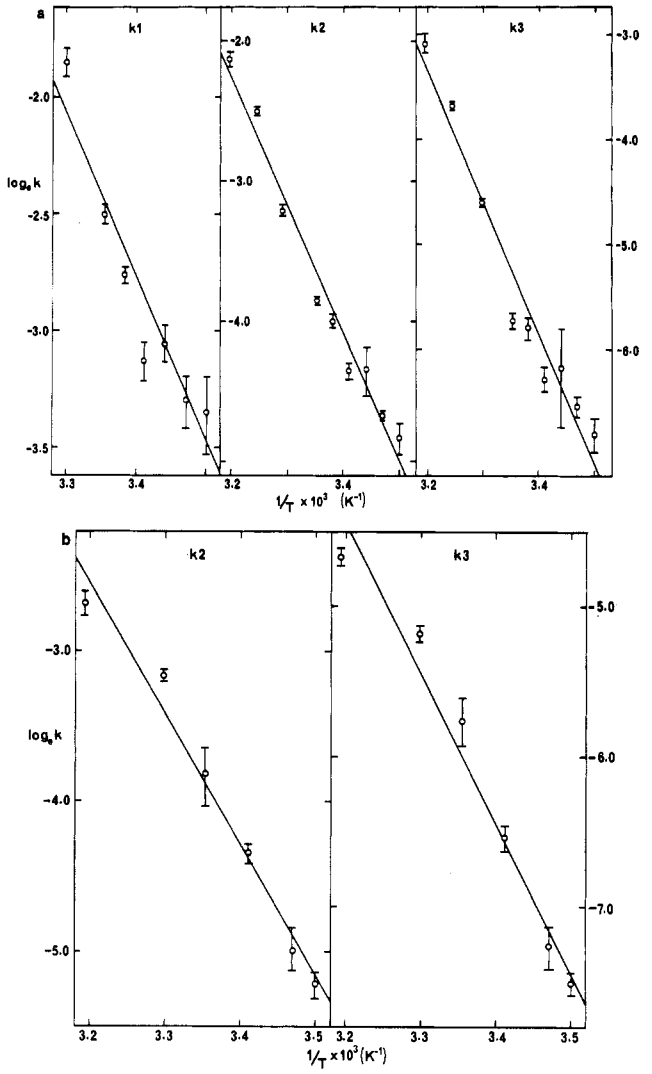

FIGURE 7: Variation with temperature of dissociation rate constants for echinomycin and calf thymus DNA at different levels of binding. The data are presented in the form of Arrhenius plots. The total calf thymus DNA concentration was 500 μM in each case. (a) $r = 0.05$. The lines fitted to the points are least-squares lines with the following parameters: k_1, intercept = 21.3 ± 3.9, slope = (7.0 ± 1.1) × 10^3; k_2, intercept = 26.9 ± 1.7, slope = (9.1 ● 0.5) × 10^3; k_3, intercept = 36.5 ± 3.2, slope = (12.4 ± 0.9) × 10^3. (b) $r = 0.015$. The least-squares lines have the following parameters: k_2, intercept = 25.3 ± 2.2, slope = (8.7 ± 0.7) × 10^3; k_3, intercept = 27.6 ± 3.2, slope = (10.0 ± 1.0) × 10^3.

τ_3. The variation of τ_1 with temperature at $r = 0.015$ is not shown because at this low level of binding the faster component, while still resolvable, did not yield results sufficiently consistent to produce a meaningful Arrhenius plot.

The activation energies at $r = 0.05$ follow the anticipated trend in that the longer time constants are associated with the highest energy values. However, the entropy of activation increases for the longer time constants, such that the tighter complex appears to have a greater relative freedom of movement in the transition state. At the lower binding ratio the activation energies are very similar to those at the higher r value and, if anything, are slightly smaller. The major difference between activation parameters at the two ratios seems to lie in the preexponential factor, which corresponds to a smaller entropy of activation at the lower level of binding. This speaks for less freedom of movement in the transition state as compared to the fully bound drug molecule.

Variation with Ionic Strength. The effect of varying the ionic strength on the dissociation times is shown in Table IV. Ionic strengths >0.5 could not be studied because the $NaDodSO_4$ precipitated on addition. It can be seen that decay times become faster at higher ionic strengths. This is consistent with the known decrease in the binding constant with increasing ionic strength (Wakelin & Waring, 1976). At $I = 0.5$ the total observed dissociation amplitude was much smaller, and only two dissociation times could be resolved. These were designated τ_2 and τ_3 since it appeared that τ_1 had become too fast to measure. It is impractical to attempt to correlate these dissociation times with measured binding constants because in all dissociation experiments the actual ionic strength must include a contribution from the 2% $NaDodSO_4$ which by itself is equivalent to an ionic strength of 0.08. However, it does appear that the variation in dissociation time constants is less pronounced than the variation in binding constants, suggesting that the association rates may also vary as a function of ionic strength. If this simple analysis is correct, then association should become slower at higher ionic strength.

Kinetics of Association between Echinomycin and DNA. Preliminary tests revealed that even with a simple technique of rapid mixing by hand it was possible to observe a certain proportion of the antibiotic–DNA association reaction. This corresponded to a time-dependent decrease in absorbance accounting for ~20% of the total change. The remaining 80%, even at the lowest temperatures investigated, occurred too fast to be measured.

The observable part of the association reaction was reasonably fitted by a single exponential. However, the residuals to the fitted curve were not always randomly distributed; thus, there is some evidence that even this reaction may contain two components which could not be properly resolved.

In order to determine whether this observable association was due to a bimolecular process, we measured the rate constant as a function of DNA concentration. For a bimolecular process

$$\text{drug} + \text{DNA} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{DNA–drug}$$

the observable rate constant, k, will be given by $k = k_{-1} + k_1[\text{DNA}]$, provided that the DNA concentration in each experiment is sufficiently high that it remains effectively unchanged throughout the course of the reaction. If the reverse reaction (dissociation) is slow compared with the forward rate, as suggested by the previous results, k_{-1} can be ignored and a plot of rate constant against DNA concentration should give a straight line passing approximately through the origin with slope k_1. The effect of DNA concentration on the rate constant at 12.5 °C is shown in Figure 8a. It can be seen that the rate

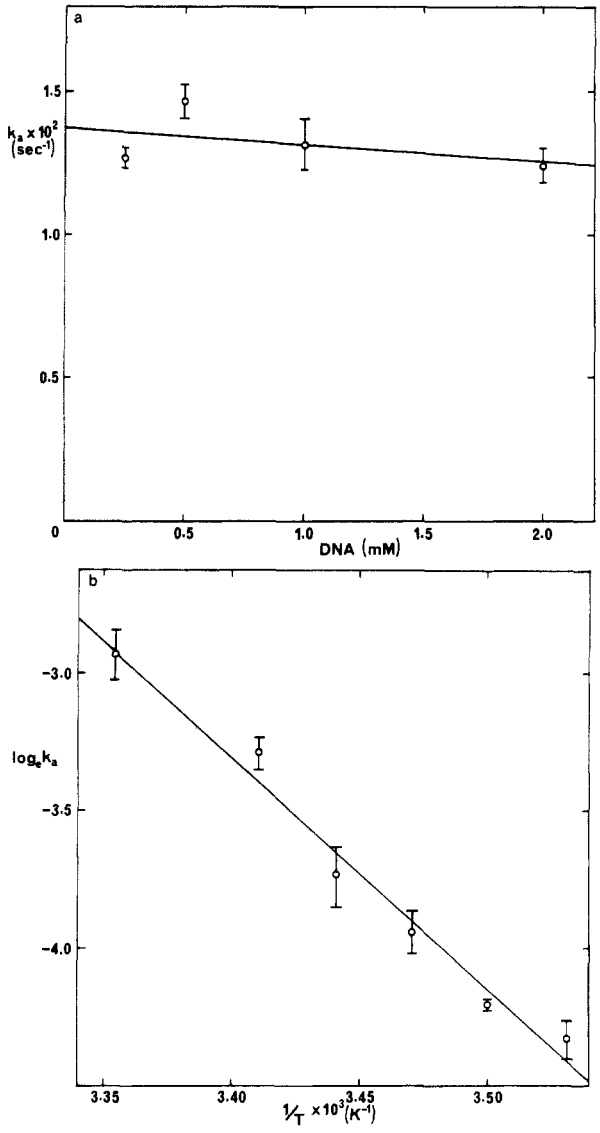

FIGURE 8: Kinetics of association between echinomycin and calf thymus DNA. (a) Dependence of the observable association rate constant on the total DNA concentration. (b) Effect of temperature on the association reaction. The data are presented in the form of an Arrhenius plot, where the ordinate represents the natural logarithm of the association rate constant and the abscissa represents the reciprocal of the absolute temperature. The straight line drawn through the points represents a least-squares fit with intercept = 25.1 ± 2.2 and slope = 8.4 ± 0.6.

constant is virtually independent of the DNA concentration, suggesting that the observable process is not a bimolecular reaction but is probably a unimolecular rearrangement, occurring after the initial process of association.

The effect of temperature on the slow association step is shown in Figure 8b in the form of an Arrhenius plot. It yields the following activation parameters: ΔE = 16.7 ± 1.2 kJ mol^{-1}; $A = 8.4 \times 10^{10}$ s^{-1}; $\Delta S^{\ddagger} = -9.6$ eu mol^{-1}. The negative entropy of activation suggests that the reaction proceeds via a transition state in which the ligand has restricted freedom of movement and is consistent with the view that the observed step represents a rearrangement of the antibiotic–DNA complex, possibly as the result of interactions between the peptide backbone and the DNA helix, similar to those suggested as corresponding to the longest association times seen with actinomycin D (Müller & Crothers, 1968).

Discussion

Origin of the Complexity of the Dissociation Reaction. The discovery of three relatively slow dissociation times for natural DNAs, akin to the behavior of the peptide antibiotic actinomycin but quite distinct from that of simple intercalators like ethidium or proflavin (Müller & Crothers, 1968; Li & Crothers, 1969; Bresloff & Crothers, 1975; Wakelin & Waring, 1980), poses a problem as regards interpretation in terms of a reasonable kinetic model. Two extreme situations can be envisaged: the existence of three parallel reactions occurring simultaneously or three sequential steps along the same kinetic pathway. The first possibility would represent, for instance, the drug dissociating from three different types of sites on the DNA, each of which is characterized by its own dissociation time. The second alternative implies that we see three distinguishable species along the dissociation pathway, such that the drug does not come off the helix in a one-step process but goes through a series of discrete intermediates. It is worth noting that for any intermediate to be detectable by our methods it must have significantly different absorbance properties from the preceding and subsequent intermediates in the pathway. Since the absorbance characteristics are largely a property of the chromophores, this means that these moieties would have to pass through different bound forms in the process of dissociation, e.g., via sequential dissociation of the chromophores involving the formation of a transient monointercalated complex. The rigidity of the peptide ring (Ughetto & Waring, 1977; Cheung et al., 1978) would be hard to reconcile with this idea. A monointercalated state is likely to be very unstable and would probably result in rapid reintercalation of the first chromophore or dissociation of the second. Also, this model cannot account for the three-exponential decay observed, since it would predict only two exponentials which should be closely coupled. On the face of it, a model involving parallel dissociation of the drug from three classes of binding sites appears to afford the best explanation of the results.

Evidence for this model comes from the experiments with synthetic DNAs, where the dissociation of echinomycin was completely described, over several half-lives, by a single exponential. This in itself is somewhat surprising, even assuming that dissociation from each different binding site yields a single-exponential decay, because alternating polymers necessarily contain two different potential tetranucleotide binding sites. If dissociation from each site is characterized by a single time constant, we would expect the dissociation from the polymer to be described by two exponentials with amplitudes corresponding to the relative occupancy of each of the tetranucleotide sequences. Since a single exponential is observed, this cannot be occurring. Two possible explanations may be considered. Either the antibiotic binds, and hence dissociates, equally well from each site so that the two decays are so tightly coupled as to be unresolvable or it is able to discriminate between the two available sites and bind exclusively to one or the other. There is no way of distinguishing between these alternatives from the present experiments. However, with actinomycin the dissociation from poly(dG-dC) is also described by a single exponential, ostensibly due to binding of the antibiotic to GC sites rather than CG (Krugh & Neely, 1973).

Further evidence comes from the effect of varying r on the relative amplitudes of the three dissociation times. If the three decays were the result of sequential reaction steps, then their relative amplitudes should remain constant, since it would be anticipated that each drug molecule would dissociate via the same kinetic pathway irrespective of the number of other molecules bound. The marked decrease of A_3 with binding

ratio (Figure 5) is clearly at variance with the sequential model but supports the view that the three time constants arise from the existence of different classes of binding sites. Our analysis suggests that the tightest binding sites saturate at an r value around 0.003, i.e., one drug molecule per 333 nucleotides. If complete saturation of the DNA occurs at $r = 0.125$ (i.e., a site size of four base pairs), then 2.4% of the total available sites are of this tight binding form. This suggests that of the 136 distinguishable permutations of four base pairs (Lee, 1977) three or four bind the drug much more tightly than the rest. It is not possible from the present data to determine which sequences correspond to each of the dissociation times, and indeed it seems likely that the treatment of dissociation curves in terms of three exponentials, while being mathematically precise, is a simplification of the real situation. Most probably the analysis of the data artifically divides the binding sites into three classes.

The variation of each time constant with r is also somewhat surprising. It appears that at low binding ratios the dissociation times themselves become much longer, the effect becoming quite pronounced at r values below 0.025. There could be several possible explanations of this phenomenon. Cooperativity in the binding of the antibiotic may be considered, but if it results from some drug-induced local distortion of the helical structure as Krugh et al. (1979) suggested for the dissociation of actinomycin from poly(dG-dC), it would be necessary to postulate that relaxation of the DNA from the perturbed state is slow compared to the dissociation of the antibiotic.

Another possibility is that by fitting what may truly be a multiexponential decay to the sum of three exponentials we observe a shift in the artificial allocation of different types of binding sites to the various τ values. This is consistent with the data for τ_1 and τ_2 since they become longer at lower r values, perhaps due to the incorporation of contributions from longer, more abundant time constants into these decays. However, this explanation does not account for the variation of τ_3 with r since, as noted earlier, the longest dissociation times are not detectable in the dissociation of higher r value complexes. Also, the value of τ_3 was found to remain essentially unchanged if the first 200–300 s of the observed decay were deleted (effectively eliminating the first two relaxation effects, which would be virtually complete by that time) and the remainder of the decay analyzed in terms of a single exponential.

One important difference between this variation of τ with natural DNA and that seen with actinomycin and poly(dG-dC) is that the dissociation from each site appears to become progressively slower at lower binding ratios, whereas for actinomycin–poly(dG-dC) complexes the dissociation is slowest for the higher ratios (Krugh et al., 1979). In one case the binding of the antibiotic appears to perturb the nucleotide structure to a form that slows the dissociation, and in the other case it is speeded up. This could be a result of the unwinding induced in the base pairs adjacent to the actual intercalation site. The magnitude of this adjacent unwinding could then determine whether the interaction of any contiguously bound ligands will be strengthened or weakened.

The relative proportion of A_3 measured for each DNA correlates quite well with its gross base composition. It appears most abundant in the higher G + C containing DNAs from *E. coli* and *M. lysodeikticus*, suggesting that this decay represents dissociation from sites rich in G + C residues. However, it is unlikely to be due to dissociation from the sequence GCGC (or CGCG) itself, since it is a factor of ~5 longer than that observed with poly(dG-dC) at a comparable binding ratio. However, nothing is known about the variation of τ with r for echinomycin and poly(dG-dC), and it is even possible that the helical conformation of the synthetic DNA is not the same as that found in natural DNAs (Leslie et al., 1980; Wang et al., 1979). If so, the dissociation from a sequence in natural DNA might have very different characteristics from that observed with the same sequence in a synthetic DNA. However, it would seem equally reasonable to suppose that the longest dissociation occurs from sites containing all four nucleotides. The observation that the value of A_3 is greatest for DNA containing 72% G + C lends weight to the suggestion that the preferred sequence(s) may contain one AT and three GC base pairs (Lee & Waring, 1978a). Despite the implied asymmetry, such sequences cannot be ruled out as candidates for the preferred binding sites of a nearly symmetrical antibiotic molecule.

Facilitated Dissociation. The dependence of dissociation times on the absolute DNA concentration was an unexpected, yet highly significant, observation. A similar effect has also been described for the dissociation of an acridine dimer from DNA by Capelle et al. (1979), which they interpreted as being due to formation of a transient ternary complex between the drug and two DNA molecules. This is reminiscent of the direct ligand transfer mechanism by which it seems that ethidium can move from one binding site to another without having to go through a free solution state (Bresloff & Crothers, 1975; Wakelin & Waring, 1980).

The model we propose to account for this phenomenon involves some interaction, albeit weak, between the back of the peptide portion of an intercalated antibiotic molecule (facing away from the helix) and another DNA molecule, similar to the concept advanced by Müller & Crothers (1968) to explain the strange viscosity effects seen with actinomycin D and calf thymus DNA. The formation of a transient ternary complex might well aid the dissociation of echinomycin from its intercalated state. This process may be termed "facilitated dissociation".

It is worth emphasizing that the phenomenon is not specific for DNA but is equally effective with RNA. Indeed, it is possible that the effect is entirely nonspecific and could be mediated by materials other than nucleic acids such as proteins.

Weak nonspecific interaction between some portion of the peptide and the DNA may be implicated in the weak binding observed for synthetic depsipeptides such as ELSERTA and ACIMBO (Lee & Waring, 1978b) for which an intercalative mode of action can be virtually ruled out. It could also explain the weak interaction which has been observed between certain quinoxalines and poly(rA)·poly(rU) (Lee & Waring, 1978b; Fox et al., 1980), since this probably does not involve intercalation. However, careful inspection of data from previous sedimentation studies (Wakelin & Waring, 1976; Lee & Waring, 1978a,b; Fox et al., 1980) has failed to reveal any evidence of intermolecular cross-linking or aggregation of DNA molecules provoked by quinoxaline antibiotics.

Association. An estimate of the expected "average" association rate constant can be made by multiplying the intrinsic binding constant $K_{(0)}$ by the dissociation rate constant. For echinomycin with calf thymus DNA at ionic strength 0.01, $K_{(0)} = 5.48 \times 10^5\ M^{-1}$ (Wakelin & Waring, 1976), and, taking k_2 at the lowest ionic strength measured as the average dissociation rate constant ($0.02\ s^{-1}$), the average association rate constant then becomes $1.1 \times 10^4\ M^{-1}\ s^{-1}$. At a DNA concentration of 500 μM the pseudo-first-order rate constant for the reaction should therefore be $\sim 5.5\ s^{-1}$ which is clearly too

fast to be measured by the techniques employed. Although these calculations obviously represent a gross simplification, they nevertheless serve to emphasize that the observed reaction is likely to be several steps removed from the primary process of association. It is again worth remembering that for any reaction to be detectable it must affect the environment of the chromophores, since these are the parts of the antibiotic molecule responsible for the absorbance in the region of 320 nm. Thus the events responsible for the observed reaction must alter the environment of the chromophores as well as maximizing any hydrogen-bond contacts between the peptide backbone and the DNA as others have postulated for the slow reactions seen with actinomycin (Bittman & Blau, 1975; Shafer et al., 1980).

Sequence Selectivity. Probably the most important achievement of the present experiments is the evidence for more than one class of intercalative binding site on the DNAs. This possibility had already been inferred from the variation in binding constants for DNA of differing base composition (Wakelin & Waring, 1976; Lee & Waring, 1978a,b). An important consequence of heterogeneity of binding sites should appear as curvature in the equilibrium binding isotherms. If the apparent $K_{(0)}$ is really a combination of several microscopic binding constants, then the Scatchard plot should rise significantly more steeply than predicted at low values of r, where the drug will preferentially bind to the tighter sites. While there has indeed been evidence of this in binding isotherms previously described, the deviations from predicted curves are concentrated in the low-r region of the curve where the data are least accurate. It is in regard to this problem that the technique of measuring dissociation kinetics is uniquely valuable. It provides a direct means of following the dissociation of a small part of the total bound ligand (i.e., τ_3) in the presence of an excess of identical molecules bound to much weaker sites. For instance, the longest time constant in the dissociation of echinomycin from calf thymus DNA represents a maximum contribution of $\sim$0.003 to the total r value. By itself the absorbance at this r value would be far too low to measure, especially in view of the vanishingly small amount of free drug present. However, in dissociation experiments it is not the absolute absorbance which is measured but the absorbance change with time, so that blanking errors are not relevant and the free drug concentration is not measured.

The only other feasible means of measuring such tight binding at low ratios involves the use of radioactively labeled antibiotic, which is not readily available. Recently, we have succeeded in synthesizing labeled echinomycin, and preliminary experiments using solvent partition analysis yield the anticipated result that the antibiotic seems to interact preferentially with tighter sites in the DNA at low levels of binding. Details will be reported elsewhere.

Acknowledgments

We thank Drs. T. R. Krugh and D. M. Crothers for helpful discussions and for providing access to unpublished data and Dr. M. Johnson for making available his computer program.

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Interaction of Crystal Violet with Nucleic Acids†

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ABSTRACT: Sedimentation and viscosity experiments reveal that when crystal violet binds to closed circular deoxyribonucleic acid (DNA) the helix becomes unwound. The average angle of unwinding per bound dye molecule is 9.8 ± 0.6°. Viscosity measurements with sonicated rodlike fragments of calf thymus DNA provide evidence that the binding causes an apparent decrease in contour length. Scatchard plots for binding to DNA are curvilinear, concave upward, indicative of strong exclusion effects and/or heterogeneity of binding sites having different affinity. By contrast, binding curves for RNA are curvilinear, concave downward, suggestive of cooperativity. Crystal violet binds preferentially to DNA, its intrinsic association constant being 15-fold lower for ribonucleic acid (RNA) at ionic strength 0.01 and 46-fold lower in 0.2 M salt solution. Heat denaturing DNA radically alters the binding mechanism such that Scatchard plots acquire the "humped" appearance characteristic of a cooperative reaction. Its preference for binding to helical DNA is confirmed by a large increase in the "melting" temperature of calf thymus DNA in buffer of low ionic strength. Raising the ionic strength decreases affinity constants generally, and at a salt concentration of 0.75 M binding to secondary sites on DNA is substantially reduced. Temperature-jump measurements reveal two fast closely coupled relaxation times at low levels of binding whereas at higher binding ratios an additional two exponential components can be resolved, again closely coupled but well separated from the fast effects. Thus, the ligand forms at least two distinct complexes with DNA at low levels of binding, and there are not less than four bound species at higher binding ratios. The visible absorption spectrum of the crystal violet–DNA complex becomes increasingly bathochromic and hypochromic as the binding ratio is raised. However, the spectrum of the dye bound to RNA is characterized by a strong hypsochromic component and a much greater hypochromism at higher binding ratios than is seen with DNA. These spectral properties are attributed to changes in the propeller-like conformation of the dye due to rotations which affect the relative disposition of the phenyl rings and/or their dimethylamino substitutents.

Studies of the interaction between triphenylmethane dyes and deoxyribonucleic acid (DNA)[1] have produced conflicting accounts of the mechanism of binding (Lerman, 1961, 1964 a,b; Neville & Davies, 1966; Armstrong & Panzer, 1972; Müller & Gautier, 1975). Lerman (1961, 1964a) reported that these dyes increase the viscosity of DNA to an extent comparable to that found for acridines, which led him to propose that both types of ligand intercalate. He also found that on binding to DNA there was a marked decrease in the reactivity of the amino groups of pararosaniline; this he offered as further evidence that the cation is at least partially buried within the helix (Lerman, 1964b). Armstrong & Panzer (1972) confirmed the increase in viscosity up to r (moles of dye bound per mole of DNA phosphate) = 0.1 and suggested that pararosaniline intercalates so as to exclude similar binding at the three nearest-neighbor sites on either side of a bound dye molecule. In addition, they identified a second form of binding at $r > 0.125$ which did not affect viscosity and which they postulated to involve external attachment to the surface of the DNA helix. In contrast to these reports, X-ray diffraction studies on pararosaniline–DNA fibers did not reveal the intercalation-induced changes in layer-line spacing observed with acridine–DNA complexes (Neville & Davies, 1966). Similarly, Müller & Gautier (1975) found only very small effects of crystal violet on the viscosity and sedimentation coefficient of sonicated rodlike fragments of DNA which, although tending in the correct direction, were considered far too small to be compatible with an intercalation process. They concluded that crystal violet binds externally and that the dye, in addition to having a general nonspecific affinity for DNA, interacts preferentially with two adjacent A-T base pairs. They further surmised that the base pair selective interaction exhibits exclusion effects whereas the nonspecific binding is cooperative (Müller & Gautier, 1975).

The crystal structure of triphenylmethyl perchlorate shows the carbonium ion as a symmetrical propeller-shaped molecule with three coplanar central bonds bearing the aromatic rings inclined at an angle of 54° to one another (Gomes de Mesquita et al., 1965). NMR evidence confirms that this propeller configuration almost certainly pertains in solution (Moodie et al., 1959; Olah, 1964; Farnum, 1964, 1967; Schuster et al., 1968). This nonplanar conformation contrasts with that of the established fused-ring aromatic intercalating agents, and it might therefore be supposed that intercalation of the carbonium ion is precluded on steric grounds. A further feature which distinguishes triphenylmethane dyes from classical in-

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[1] Abbreviations used: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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