Stopped-Flow Kinetic Studies of Actinomycin Binding to DNAs†

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ABSTRACT: Stopped-flow kinetic studies of the association of actinomycins with natural and synthetic DNA duplexes are presented. The actinomycins examined were D (C_1), D lactam (in which the pentapeptide rings are closed by lactam instead of lactone linkages), X_2, $X_{0\beta}$, and actinomine. The DNAs used included calf-thymus DNA, PM2 DNA, and two synthetic d(A-T)-like copolymers containing 2,6-diaminopurine (DAP) in place of adenine residues, poly[d($\overline{\text{DAP}}$-T)]·poly[d($\overline{\text{DAP}}$-T)] and poly[d($\overline{\text{DAP}}$-A-T)]·poly[d($\overline{\text{DAP}}$-A-T)]. Apparent equilibrium constants indicate that the DAP-containing polynucleotides bind actinomycin strongly. Complex formation of actinomycins D, D lactam, X_2, and $X_{0\beta}$ with these DNAs can be deconvoluted into five rate processes. These steps do not necessarily proceed to completion. The rates of two of these steps display a first-order dependence on DNA concentration. The large negative entropies of activation of these steps suggest a high degree of restriction to freedom of motion in the respective transition states. The rates of the remaining three steps are independent of DNA concentration. Kinetic parameters of actinomycin binding to DNAs are presented and suggestions are made about some of the molecular events believed to be responsible for the appearance of the five rate processes. For example, for DNA, poly[d($\overline{\text{DAP}}$-A-T)], and poly[d($\overline{\text{DAP}}$-T)], the observed order of apparent second-order rate constants, normalized to the concentration of actinomycin binding sites, suggests that binding of the antibiotic occurs most rapidly at binding sites (G-C or d$\overline{\text{DAP}}$-T) near d(A-T) base pairs, where weakening of the double-helical conformation requires the least energy. Results obtained from studies of actinomycin D binding to heat-denatured poly[d($\overline{\text{DAP}}$-A-T)] and of actinomine and actinomycin D lactam binding to DNA suggest that the slow rate processes are related to an actinomycyl-pentapeptide-induced unwinding of the sugar–phosphate backbone of DNA accompanying insertion of the cyclic peptides into DNA.

A wealth of information has been compiled concerning the interaction of actinomycin D (C_1) with DNA. Chemical studies led to the proposal that the actinomycin chromophore, phenoxazone, binds in the minor groove of helical DNA, where specific hydrogen-bonded interactions can occur between the phenoxazone quinone oxygen atom and the 2-amino group of guanine, between the phenoxazone amino group and the guanine N-3 atom, and between the phenoxazone amino group and the furanose oxygen atom of deoxyguanosine (Hamilton et al., 1963; Cerami et al., 1967). Another outside-binding model was proposed from studies with space-filling models (Gursky, 1970). It was suggested that one peptide lactone ring of actinomycin binds in the minor groove of DNA; formation of two specific hydrogen bonds between the externally bound phenoxazone ring and DNA results in uncoiling of the helix; the change in helix rotation allows the second peptide lactone ring to bind in the minor groove, with the two pentapeptide rings of bound actinomycin oriented in trans positions with respect to the phenoxazone chromophore. Spectroscopic, hydrodynamic, and kinetic studies of the interaction of actinomycin and actinomycin analogs with sonicated DNA (Müller and Crothers, 1968) and investigation of the sedimentation of the superhelical DNA in the presence of actinomycin (Waring, 1970) led to the proposal that the phenoxazone is intercalated adjacent to a G-C base pair. The three-dimensional structure of a 1:2 complex formed between bromoactinomycin and deoxyguanosine was solved and the configuration obtained from the X-ray crystal analysis was used to propose a detailed model for the actinomycin–DNA complex (Sobell and Jain, 1972). In this model the phenoxazone ring is intercalated between G-C and C-G base pairs (where guanine residues are on opposite strands) and a strong hydrogen bond is formed between the guanine 2-amino group and the carbonyl oxygen atom of the L-threonine residue of the cyclic peptide in the minor groove. Additional stabilization is derived from hydrophobic interactions between groups on the pentapeptides and sugar residues and from specific weaker hydrogen bonds. Several recent spectral studies with mono-, di-, and hexanucleotides have concluded that important features of the Sobell–Jain stereochemical model exist in solution, such as a strong nucleotide–peptide intermolecular hydrogen bond and stacking of the phenoxazone and guanine rings (Krugh, 1972; Schara and Müller, 1972; Krugh and Neely, 1973; Patel, 1974).

We performed kinetic measurements of the interaction of actinomycin and several actinomycin analogs with native and heat-denatured calf-thymus DNA, superhelical DNA, and synthetic polydeoxyribonucleotides. In addition to lactone analogs of actinomycin the lactam analog of actinomycin D shown in Figure 1 was used. It was synthesized and shown to possess biological activity by Meienhofer and Patel (1971). Cerami et al. (1967) produced two d(A-T)-like polymers with partial or complete substitution of adenine by 2,6-diaminopurine (DAP)[1] and found that intro-

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[1] Abbreviations used are: DAP, 2,6-diaminopurine; poly[d($\overline{\text{DAP}}$-T)], a deoxyribonucleotide copolymer with an alternating sequence of 2,6-diaminopurine and thymine residues; poly[d($\overline{\text{DAP}}$-A-T)], a d(A-T)-like polymer with adenine/DAP mole ratio = 10; CT-DNA, calf-thymus DNA; T_m, melting temperature (50% of total hyperchromicity).

duction of DAP into poly[d(A-T)] resulted in actinomycin sensitivity. Since actinomycin does not interact with synthetic poly[d(A-T)]·poly[d(A-T)] (Goldberg et al., 1962), the deoxynucleotide copolymers, poly[d($\overline{\text{DAP}}$-A-T)]·poly[d($\overline{\text{DAP}}$-A-T)] (in which 10 mol % of the adenine residues has been replaced by DAP) and poly[d($\overline{\text{DAP}}$-T)]·poly[d($\overline{\text{DAP}}$-T)], seemed appealing for kinetic investigations of actinomycin binding because they have defined a homogeneous binding sites. The results of the kinetics of association of actinomycins with DNAs and a discussion of the possible molecular nature of some of the rate processes that are observed are described here.

Experimental Section

Materials. Actinomycin D was obtained from Merck Sharp and Dohme, Inc. [1′,1′-Bis(L-α,β-diaminopropionic acid)]-actinomycin D lactam (actinomycin lactam) was a gift from Dr. Johannes Meienhofer and Dr. Eric Atherton. Actinomine was a gift from Dr. Edward Reich. Concentrations of solutions of antibiotics were determined spectrophotometrically using the following extinction coefficients: actinomycins D, X_2, $X_{0\beta}$, and lactam, 24,450 M^{-1} cm^{-1} at 440 nm; and actinomine, 22,500 at 445 nm.

Calf-thymus (CT) DNA was purchased from Worthington Biochemical Corp. DNA was dissolved in 0.02 *M* NaCl solution by rotation at 4° at 2 rpm. Solutions were clarified by centrifugation at 14,000 rpm and dialyzed against buffer solution. Poly[d(A-T)], poly[d($\overline{\text{DAP}}$-A-T)], poly[d($\overline{\text{DAP}}$-T)], poly(dI), poly[d(G-C)], and SV 40 DNA were generously supplied by Drs. Edward Reich and Robert Klett. The syntheses of the copolymers were performed by Dr. Klett as follows. For poly[d($\overline{\text{DAP}}$-T)], the reaction mixture, per milliliter of 0.07 *M* potassium phosphate buffer containing 0.005 *M* Mg^{2+} (pH 7.0), consisted of 500 nmol of DAP-deoxynucleoside triphosphate (d$\overline{\text{DAP}}$TP), 500 nmol of thymidine triphosphate (dTTP), 10–20 nmol of poly[d($\overline{\text{DAP}}$-T)], 10–15 units of DNA polymerase (specific activity 15,000–17,000 units/mg of protein), and 100 μg of bovine serum albumin (to protect DNA polymerase from denaturation). For poly[d($\overline{\text{DAP}}$-A-T)], 450 nmol of deoxyadenosine triphosphate (dATP) and 50 nmol of d$\overline{\text{DAP}}$TP per ml were used in place of 500 nmol/ml of d$\overline{\text{DAP}}$TP, and 10–20 nmol/ml of poly[d(A-T)] was used as primer in place of poly[d($\overline{\text{DAP}}$-T)]. The reactions were monitored by addition of [α-^{32}P]d$\overline{\text{DAP}}$TP and [^{3}H]dTTP or [^{3}H]dATP to a small aliquot of the reaction mixture. The amount of acid-insoluble polymer was determined after adding cold 5% trichloroacetic acid and pouring the reaction mixture through a Millipore filter. Phenol that had been equilibrated with buffer was added to stop the reaction. The aqueous phase was dialyzed extensively against 0.01 *M* Tris buffer containing 1 *M* NaCl, then against 0.01 *M* Tris buffer containing 0.1 *M* NaCl, and finally against 0.01 *M* phosphate buffer (pH 7.0). Poly[d(G-C)] was synthesized in 0.07 *M* glycine buffer containing 0.005 *M* Mg^{2+} (pH 9.2), using 500 nmol/ml each of deoxycytidine triphosphate (dCTP) and deoxyguanosine triphosphate (dGTP), 50 nmol/ml of poly[d(G-C)], 30 units/ml of DNA polymerase, and 100 μg/ml of bovine serum albumin. The reaction was monitored with [^{3}H]dCTP and [^{14}C]dGTP. The copolymer contained equal amounts of deoxyguanylic and deoxycytidylic acids. The rest of the procedure was the same as that used for synthesis of the d(A-T)-like copolymers. Superhelical PM2 DNA was a gift from Dr. Michael J. Waring. The preparation contained approximately 87% closed circular molecules as estimated by sedimentation velocity. Concentrations of DNA solutions in terms of nucleotide phosphorus were determined spectrophotometrically using the following values for ε(P) at 260 nm: CT DNA, 6600 M^{-1} cm^{-1}; poly[d(A-T)], 6800; poly[d($\overline{\text{DAP}}$-A-T)], 6500; and poly[d($\overline{\text{DAP}}$-T)], 6500.

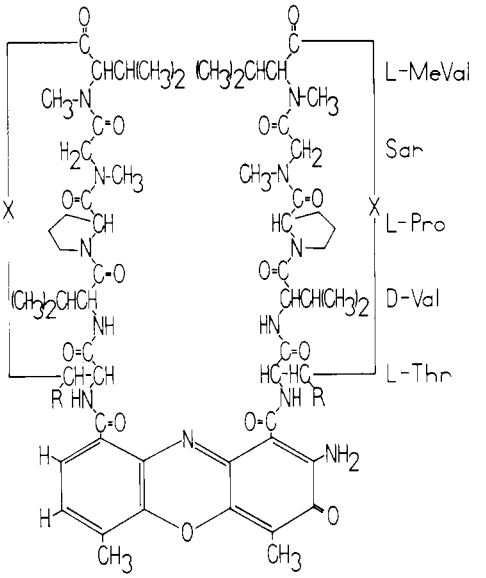

FIGURE 1: Structures of actinomycin D (X = O; R = CH_3) and [1′,1′-bis(L-α,β-diaminopropionic acid)]-actinomycin D lactam (X = NH; R = H).

Solutions were prepared in 0.01 *M* potassium or sodium phosphate buffer (pH 7.0), unless otherwise noted.

Heat-denatured CT-DNA was prepared by heating a DNA solution of known concentration at 100° for 15 min and then cooling it rapidly at 0°. Hyperchromicities of at least 30% were obtained. In 0.001 *M* potassium phosphate buffer, the hyperchromicities of poly[d($\overline{\text{DAP}}$-A-T)] and poly[d($\overline{\text{DAP}}$-T)] were approximately 40%.

Methods. ABSORPTION MEASUREMENTS. Absorption spectra were measured on a Cary 14 spectrophotometer. Spectral titrations were carried out at 25° using an expanded (0–0.1) slidewire and a cell of 1-cm pathlength and 5.5-ml capacity. The concentration of polynucleotide was maintained constant in the titrations. A stock solution consisting of the antibiotic and polynucleotide was prepared. A solution of the polynucleotide, prepared at a concentration identical with the polynucleotide concentration of the stock solution, was placed in the cell. Aliquots of the stock solution were added by micropipet. Absorption measurements were made after a period of time sufficient to assure attainment of equilibrium. During the interval between spectral measurements, the cell containing the solution was stored in the dark. Spectral titrations with actinomycin and actinomycin lactam were carried out at 425 nm.

The concentration of bound actinomycin, c_B, was calculated using the equation $c_B = (\epsilon_F c_T - A)/(\epsilon_F - \epsilon_B)$, where ϵ_F and ϵ_B are the molar extinction coefficients of free actinomycin and of actinomycin bound to the polynucleotide under investigation, respectively, A is the absorbance of the solution in the cell, and c_T is the total actinomycin concentration. To determine ϵ_B, aliquots of an actinomycin solution were added to a concentrated solution of DNA in a 1-cm pathlength cell; after addition of the initial several aliquots, the molar ratio of DNA(P) to actinomycin was typically about 500–1000. The extinction coefficient of bound

Table I: Association Constants and Number of Binding Sites for Actinomycin–DNA Interactions.

		K_{app} (M^{-1}) $\times 10^{-6}$	Nucleotides per Site
Actinomycin	DNA ___	4.21 ± 0.40	14.3
Actinomycin	Poly[d(DAP-A-T)]	2.11 ± 0.51	28.6
Actinomycin	Poly[d(DAP-T)]	5.56 ± 0.67	6.0
Actinomycin lactam	DNA	1.42 ± 0.04	9.1
Actinomycin lactam	PM2 DNA	2.39 ± 0.11	13.0

actinomycin was determined either by dividing the measured absorbance by the actinomycin concentration, or by extrapolating a plot of ϵ vs. actinomycin concentration to the limiting value of ϵ at low actinomycin concentration. Values of ϵ_B were determined in each spectral titration; for actinomycin bound to DNA, PM2 DNA, poly[d(DAP-A-T)], or poly[d(DAP-T)] the range of ϵ_B was about 13,000 ± 1000 M^{-1} cm^{-1}, and for actinomycin lactam bound to DNA and PM2 DNA, ϵ_B was about 10,300 M^{-1} cm^{-1}.

The apparent equilibrium constant, K_{app}, and the number of binding sites, n, were obtained from the Scatchard (1949) equation, $r/c = K_{app}(n - r)$, by plotting the ratio r/c vs. r; r is the molar ratio of bound actinomycin to the total DNA(P) concentration and c is the molar concentration of free actinomycin. The latter is equal to the difference between c_T and c_B. A computer program was developed to fit points to one or two least-squares fit lines by comparing mean square deviations. The results shown in Table I and Figure 2 represent the computer analysis of the straight line for the strong-binding process, i.e., data obtained at low values of r. The error limits reported for K_{app} were calculated from the range of the slope with confidence levels of 50% using a student t test. The error associated with the number of nucleotides per site is approximately ±10%.

KINETIC MEASUREMENTS. Stopped-flow studies were conducted with a Durrum-Gibson stopped-flow spectrophotometer equipped with a Tektronix 564 storage oscilloscope and Polaroid camera. The temperature was maintained constant with a Lauda K2-R temperature circulator. The temperature of the stopped-flow experiments was 25.0°, and the final concentration of actinomycin D or actinomycin analogs was 7.5 μM unless stated otherwise. The concentration of DNA was varied from about 135 to 400 μM. A total signal of 800 mV and a slit width of 1 mm were used. Equal volumes of actinomycin and polynucleotide were mixed rapidly, and the change in transmittance at 425 nm was recorded on the oscilloscope screen. An electric timer was started at the initiation of the mixing and the kinetics of the slowest reaction were monitored by marking the position of the light spot on the oscilloscope screen at different times (see Figure 3). Rate constants were calculated from analysis of several relaxation curves taken at different time settings. Errors obtained from least-squares plots of kinetic data are expressed at a 90% confidence level. Because of the difficulty in separating relaxation curves whose time constants differ by less than a factor of 10, there is an error of as much as ca. 20% associated with each rate constant and transmittance change.

It was found that the molecular weight of CT-DNA decreased on passing through the stopped-flow apparatus. Therefore, prior to use in kinetic experiments with actinomycin, the DNA was sheared by filling both drive syringes with DNA solution and passing it through the stopped-flow apparatus a total of five times. By this procedure the molecular weight of DNA reached a value that was not diminished appreciably further when mixed in the stopped-flow apparatus. Viscosity measurements made with a Zimm-Crothers type viscometer indicated that the molecular weights of the original and five-times sheared DNA were approximately 9×10^6 and 4×10^6, respectively.

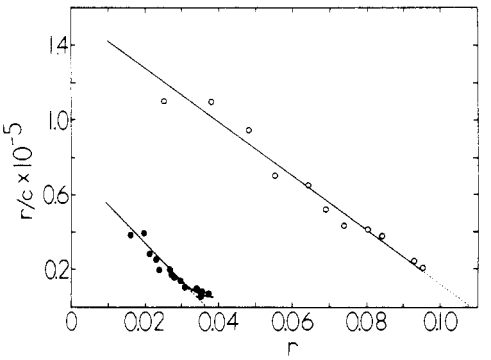

FIGURE 2: Scatchard plots of binding of actinomycins to DNAs: (●) actinomycin binding to poly[d(DAP-A-T)]; (○) actinomycin lactam binding to DNA. r is the molar ratio of bound actinomycin to nucleotide phosphorus residues; c is the molar concentration of free actinomycin. The straight line represents the binding of the antibiotic to the class of high affinity binding sites.

The DNA used in the experiments described in this paper was CT-DNA unless otherwise noted.

Results

Spectral Titrations. The binding of actinomycin to natural and synthetic DNAs has been thoroughly investigated by optical titration and equilibrium dialysis techniques (e.g., Reich and Goldberg, 1964; Gellert et al., 1965; Müller and Crothers, 1968; Wells and Larson, 1970). Figure 2 shows typical binding isotherms obtained by optical titrations of actinomycin with poly[d(DAP-A-T)] and of actinomycin lactam with DNA. The apparent equilibrium constant for association, K_{app}, is the negative of the slope of the linear region of the binding isotherm; the number of binding sites per nucleotide is the intercept on the abscissa. Table I summarizes our measurements of actinomycin binding to DNA and the d(A-T)-like copolymers and of actinomycin lactam binding to DNA and PM2 DNA. The apparent equilibrium constants do not differ markedly for the various DNAs investigated. Furthermore, actinomycin lactam binds to DNA approximately as firmly as the lactone. It is also of interest that the d(A-T)-like copolymers containing 2,6-diaminopurine bind actinomycin strongly. A previous study showed, qualitatively, that a stable complex is formed between actinomycin and three synthetic polymers, as measured by changes in the buoyant density, thermal stability, and template function of the polynucleotide, and by changes in the absorption spectrum of actinomycin (Cerami et al., 1967). The d(DAP-T) copolymer binds actinomycin somewhat more tightly than DNA, and also binds more actinomycin; the polynucleotide containing 10 mol % DAP in place of adenine residues binds actinomycin less tightly than DNA, and binds less antibiotic. Since actinomycin-binding ability of a polydeoxyribonucleotide is apparently a sensitive function of its secondary structure (Wells and Larson, 1970), such a difference may arise because of a specific difference in helical configuration between poly[d(DAP-T)] and DNA in the vicinity of the actinomycin binding site.

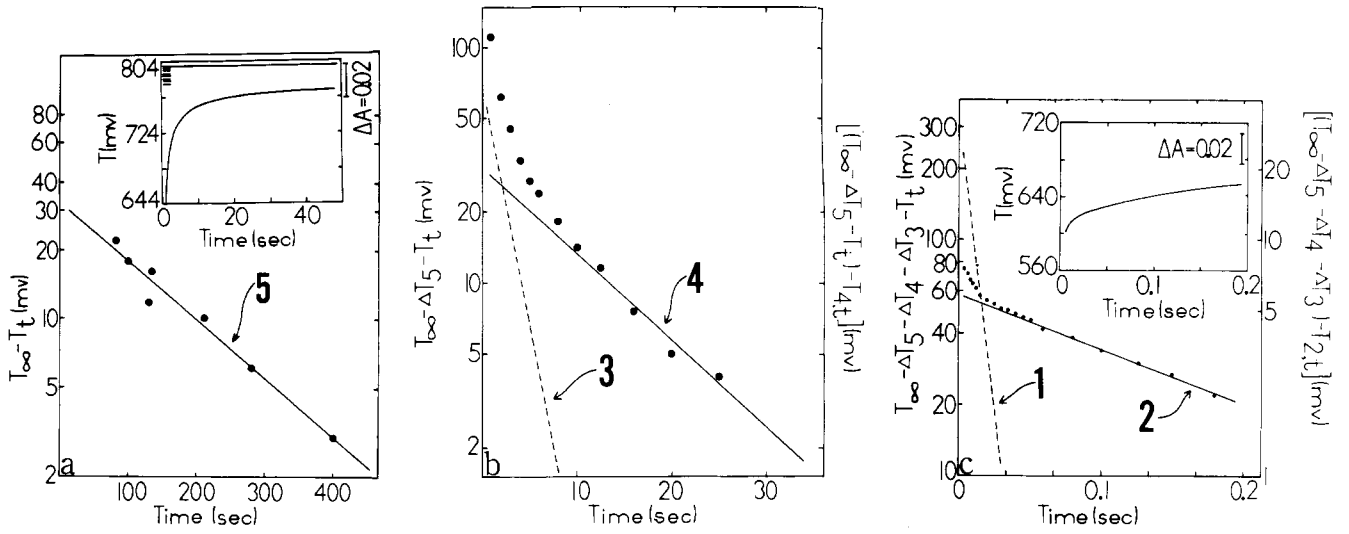

FIGURE 3: Typical traces and kinetic analyses of rate processes 1-5 of the actinomycin D-DNA association as a function of time. Actinomycin D (7.5 μM) was mixed with an equal volume of 0.15 mM of DNA in the stopped-flow spectrophotometer. The vertical axis is the transmittance at 425 nm. The horizontal axis is time in seconds. (a) Inset: A trace of transmittance (T) vs. time. The horizontal marks in the upper left corner show the transmittance (T_t) observed at the following times: 80, 100, 130, 170, 210, 280, and 400 sec. The line at 800 mV corresponds to the transmittance at the end of the reaction, T_∞. The bar in the upper right-hand corner gives the magnitude of the absorbance change; the latter is calculated from log [800/(800 − x)], where x is a given transmittance. The figure shows that a semilogarithmic plot of the transmittance differences between 800 mV and these T_t values vs. time gives a straight line. Rate constant k_5 is calculated from the slope of the line and the change in transmittance, ΔT_5, associated with this step is obtained by extension of the line to zero time. (b) Transmittance values (T_t) read from traces recorded at 2 or 5 sec/division are subtracted from the value of $T_\infty - \Delta T_5$. The resulting values of $T_\infty - \Delta T_5 - T_t$ are plotted on semilogarithm paper vs. time. A reaction curve having a linear portion (labeled 4) at long times is obtained. The slope of this line gives k_4. Extension of the line to zero time gives ΔT_4, the change in transmittance associated with the fourth rate process. The differences between the points in the first part of the reaction curve and the extended line are calculated. A semilogarithmic plot of the resulting values of $[(T_\infty - \Delta T_5 - T_t) - T_{4,t}]$ vs. time gives a straight line (labeled 3). ($T_{4,t}$ is the transmittance of points lying on the straight line labeled 4 at various times.) From the slope of line 3 (a more expanded scale than the one shown is used), k_3 is obtained. Extension of the line to zero time gives ΔT_3, the change in transmittance associated with the third rate process. The left-hand axis ($T_\infty - \Delta T_5 - T_t$) refers to line 4, and the right-hand axis $[(T_\infty - \Delta T_5 - T_t) - T_{4,t})]$ refers to line 3. (c) Inset: A trace of transmittance (T) vs. time recorded at 20 msec/division. The magnitude of the absorbance change is indicated by the bar in the upper right-hand corner. The transmittance values associated with steps 1 and 2 are obtained by measuring the differences between $T_\infty - \Delta T_5 - \Delta T_4 - \Delta T_3$ and T_t. The figure shows that a semilogarithmic plot of $T_\infty - \Delta T_5 - \Delta T_4 - \Delta T_3 - T_t$ vs. time gives a reaction curve having a linear portion (labeled 2) at long times. The slope of the line gives k_2. Extension of the line to zero time gives ΔT_2, the change in transmittance associated with the second rate process. The differences between the points in the curved portion and the extended line are calculated. A semilogarithmic plot of the resulting values of $[(T_\infty - \Delta T_5 - \Delta T_4 - \Delta T_3 - T_t) - T_{2,t}]$ vs. time gives a straight line (labeled 1). ($T_{2,t}$ is the transmittance of points lying on the straight line labeled 2 at various times.) Rate constant k_1 is calculated from the slope of the line. The change in transmittance associated with this reaction step, ΔT_1, is obtained by extension of the line to zero time. The left-hand axis ($T_\infty - \Delta T_5 - \Delta T_4 - \Delta T_3 - T_t$) refers to line 2, and the right-hand axis $[(T_\infty - \Delta T_5 - \Delta T_4 - \Delta T_3 - T_t) - T_{2,t}]$ refers to line 1.

Table II: Pseudo-First-Order Rate Constants and Transmittance Changes of Rate Processes for Association of Actinomycins with DNA.[a]

Actinomycin	k_1	% ΔT_1	k_2	% ΔT_2	k_3	% ΔT_3	$10^2 k_4$	% ΔT_4	$10^3 k_5$	% ΔT_5
D	75 ± 30	5	3.0 ± 0.3	28	0.39 ± 0.02	36	9.2 ± 1.5	20	7.5 ± 1.0	11
$X_{0\beta}$	152 ± 20	8	4.4 ± 0.1	23	0.50 ± 0.15	23	8.1 ± 1.2	27	6.4 ± 0.2	19
X_2	230	9	4.9	35	0.53 ± 0.13	18	8.0 ± 1.5	19	6.6 ± 0.9	19
Lactam	71 ± 20	6	3.4 ± 1.0	23	0.16 ± 0.07	17	2.0 ± 0.8	16	2.7 ± 0.2	38
Actinomine	430	57	26.5	43						

[a] The ratio of DNA(P)/actinomycin was 18. Rate constants are reported in sec^{-1}. The percent transmittance change, % ΔT_i, is the ratio of the transmittance change of a given rate process to the total change in transmittance, ΔT_{1-5}, × 100.

Kinetics of Association of Actinomycins with DNA. The formation of the actinomycin-polydeoxyribonucleotide complex(es) was also studied by stopped-flow kinetics. Figure 3 shows examples of the time course of complex formation. The method of analysis of the forward rate constants and transmittance changes in terms of a five-step process is described in the caption. In the presence of DNA, actinomycin lactam, X_2, and $X_{0\beta}$ underwent spectral changes similar to those found with actinomycin D. In an attempt to establish relationships between the kinetic steps and molecular events that accompany binding, a study was made of the interaction of these analogs of actinomycin D with DNA. A very fast and a very slow rate process, together with three processes of intermediate time constants, were detected by stopped-flow spectrophotometry. The kinetic data and transmittance changes calculated for each step are shown in Table II. Pseudo-first-order rate constants were calculated at low concentrations of bound actinomycin per DNA(P) in order to minimize complications that could arise if binding sites having weaker affinity were occupied. The pseudo-first-order rate constants for steps 1 and 2 and the transmittance changes associated with these processes are very similar for the actinomycin D-DNA and actinomycin lactam-DNA reactions. In the lactam reaction, rate processes 3, 4, and 5 proceed more slowly and have a different distribution of transmittance changes. Circular dichroism spectra (Blau

Table III: Dependence of Pseudo-First-Order Rate Constants on DNA Concentration.[a]

Concn of DNA (μM)	DNA(P)/Act-D	k_1	k_2	k_3	10^2k_4	10^3k_5
135	18	75 ± 30	3.0 ± 0.3	0.39 ± 0.02	9.2 ± 1.5	7.5 ± 1.0
135	18	80 ± 10	3.6 ± 1.0	0.36 ± 0.04	9.2 ± 1.5	7.5 ± 1.0
135[b]	18	68	3.6	0.47	9.6	8.8 ± 1.5
350[c]	18	140	3.3	0.37 ± 0.03	8.1 ± 1.5	6.7 ± 1.0
200	27	122	7.8 ± 0.4	0.40	8.2 ± 1.0	6.3 ± 1.0
230	31	111 ± 10	7.0 ± 0.3	0.45 ± 0.05	10.9 ± 1.0	7.3 ± 0.5
260	35	200	10.0 ± 1.0	0.35 ± 0.05	7.5 ± 1.0	6.6
370	50	174	13.7 ± 1.4	0.40 ± 0.10	8.7 ± 2.0	7.6 ± 1.1

[a] Rate constants are reported in sec^{-1}. The percent transmittance changes of rate processes 1–5, which did not vary markedly with DNA concentration, were 8 ± 2, 25 ± 5, 30 ± 5, 20 ± 5, and 15 ± 5, respectively. [b] Sodium phosphate buffer, 0.01 *M*, pH 7.9. [c] The final concentration of actinomycin D was 19.5 μM. To allow a comparison to be made of the rate data obtained from different preparations of DNA solution, data are given for two solutions of DNA (135 μM) in 0.01 *M* sodium phosphate buffer (pH 7.0).

Table IV: Activation Parameters for the Actinomycin D–DNA Interaction.[a]

Rate Process	$\Delta H^{\ddagger}$ (kcal mol^{-1})	$\Delta S^{\ddagger}_{298}$ (eu)
k_1	4.0 ± 1.0	−38.2
k_2	9.9 ± 1.4	−24.2
k_3	13.6 ± 0.3	−17.3
k_4	14.2 ± 0.9	−17.7
k_5	13.4 ± 1.3	−26.0

[a] The enthalpy of activation was calculated from semilogarithmic plots of the forward rate constants vs. reciprocal temperature. $\Delta S^{\ddagger}$ was assumed to be independent of temperature over the temperature range used (290–310 K). Parameters were calculated by the method of least squares. The ratio of DNA(P)/actinomycin was 31.

and Bittman, 1975) suggest that uncomplexed actinomycin D and actinomycin lactam have different molecular conformations, e.g., they differ in the conformations of their pentapeptide rings and/or in the orientations of the pentapeptide rings relative to the phenoxazone ring. The kinetic data shown in Table II for actinomycin D and actinomycin lactam suggest that the last three reaction steps involve changes associated with the interaction of the cyclic peptides with DNA. (It should be emphasized that in order to be detected, these changes must also affect the chromophore.) A number of factors may be responsible for the slower reactions observed with the lactam. For example, the cyclic pentapeptides of the lactone and lactam may differ in their gross conformation and in their conformational flexibility; the configurations of some of the peptide bonds may differ, e.g., at the propionate position in the lactam compared with the threonine residue in the lactone (note that this is not an isosteric substitution).

Kinetics of Actinomine Binding. The conclusion that the three slowest reaction processes involve the cyclic pentapeptides of actinomycin is supported by the results obtained for the interaction of actinomine with DNA (Table II). The structure of actinomine differs from that of actinomycin in that each large pentapeptide ring is replaced by an *N,N*-diethylaminoethyl group. The slow rate processes are absent in the binding of this peptide-lacking analog to DNA. The absence of the three slowest rate processes in the binding of actinomine to DNA suggests that the slow reaction processes correspond to accommodation of the peptide rings into binding sites of DNA. These interactions are not sensitive to substitution of a keto or hydroxyl group for hydrogen in one proline residue since the reactions represented by k_3, k_4, and k_5 are unaffected by substitution of a 3-oxo group (actinomycin X_2) or a 3-hydroxyl group (actinomycin $X_{0\beta}$) in one L-proline residue (Table II).

The order of biological activity of the actinomycins is $X_2 > D > X_{0\beta}$ (Reich et al., 1962). It has been proposed that it is the slow dissociation rate of biologically active, peptide-containing actinomycins that prevents progression of DNA-dependent RNA polymerase along the template (Müller and Crothers, 1968). The similarity of the kinetic parameters for association of actinomycin D, X_2, and $X_{0\beta}$ with DNA (Table II) suggests that their differing biological activities may result from differences in the kinetics of dissociation of the complexes they form with DNA.

Concentration Dependence. The DNA concentration dependence of the forward rate processes is shown in Table III. The reaction steps represented by k_1 and k_2 are dependent on DNA concentration, whereas those represented by k_3, k_4, and k_5 are not. Least-squares analysis of a plot of the pseudo-first-order rate constants, k_1 and k_2, vs. the sum of the concentrations of free DNA and actinomycin gave apparent second-order rate constants of $3.75 \pm 0.95 \times 10^5$ and $4.41 \pm 0.58 \times 10^4$ M^{-1} sec^{-1} for k_1 and k_2, respectively. The molarity is based on the concentration of nucleotide phosphorus residues. In the Discussion section, these apparent bimolecular rate constants, normalized to the concentration of actinomycin binding sites, are compared with the rate constants calculated for actinomycin D binding to the d(A-T)-like copolymers and actinomine binding to DNA.

Temperature Dependence. The temperature dependence of the association was studied at six temperatures between 17 and 37°. The activation parameters derived from the temperature dependence of each of the kinetic constants are shown in Table IV. The large negative entropies are striking. Since values of near −40 eu are found when there is a great deal of restriction in the transition state (Page and Jencks, 1971), the very large negative entropy of activation of the first step suggests that actinomycin is bound firmly even in the first step. Thus, the reactants interact fairly specifically in the transition state of the first reaction step. An alternative explanation involving ordering of water molecules on complexation is considered unlikely because a small-angle X-ray scattering study showed that water is lost from DNA when actinomycin is bound (Zipper et al., 1972). The finding that the activation entropies are negative is not inconsistent with the equilibrium results that $\Delta S°$ is +31 eu/mol of actinomycin D bound to DNA (Gellert et al., 1965) because transition states need not resemble equilibrium processes.

Binding to Synthetic and Superhelical DNAs. The bind-

Table V: Pseudo-First-Order Rate Constants and Transmittance Changes of Rate Processes for Association of Actinomycin D with DNAs.[a]

DNA	DNA(P)/ Act-D	k_1	% ΔT_1	k_2	% ΔT_2	k_3	% ΔT_3	10^2k_4	% ΔT_4	10^3k_5	% ΔT_5
CT, native	31	110 ± 9	10	7.0 ± 0.3	28	0.45 ± 0.05	30	10.9 ± 1.0	20	7.3 ± 0.5	12
CT, heat-denatured	31	210 ± 10	36	27 ± 4	24	0.42 ± 0.08	12	8.6 ± 1.4	15	9.7 ± 0.6	13
CT, native	18	80 ± 10	5	3.6 ± 1.0	25	0.36 ± 0.04	36	9.2 ± 1.5	21	7.5 ± 1.0	13
Poly[d(DAP-T)]	15	87 ± 12	5	2.2 ± 0.3	50	0.41 ± 0.05	28	7.6 ± 2.3	9	9.4 ± 1.7	16
Poly[d(DAP-A-T)]	18	110	5	3.9 ± 0.3	22	0.36 ± 0.02	51	7.8 ± 0.6	23	8.2 ± 2.0	4
Poly[d(DAP-A-T)][b]	10	85	28	1.8	60	0.15	12				
PM2	18			8.1 ± 0.5	22	0.55 ± 0.08	32	7.3 ± 4.0	28	7.9 ± 2.0	17

[a] Rate constants are reported in sec^{-1}. [b] 1.0×10^{-3} *M* potassium phosphate buffer (pH 7.0) at 36.0°.

ing of actinomycin D to DNA and to the synthetic DNAs is characterized by very similar rate constants at comparable nucleotide/actinomycin molar ratio (Table V). It is of interest that poly[d(DAP-T)], which in addition to having defined and homogeneous binding sites for actinomycin and a perfectly regular structure, yields multiple slow association time constants. Poly[d(DAP-A-T)] has, on the average, one binding site for actinomycin per helical turn and therefore may also be considered to possess complete homogeneity of binding sites. The fact that the slow rate processes observed in the binding of actinomycin to the synthetic d(A-T)-like copolymers are identical with those observed with DNA indicates that these reactions do not arise from the heterogeneity of structure, sequence, or configuration of native DNA. As was observed with DNA, the reaction steps designated by k_1 and k_2 were dependent on the concentration of the synthetic polydeoxyribonucleotides, whereas the rate constants of the other three steps were independent of nucleotide concentration. For poly[d(DAP-T)], the apparent second-order constants, k_1 and k_2, based on nucleotide phosphorus concentration, were $5.5 \pm 2.5 \times 10^5$ and $1.7 \pm 0.3 \times 10^4$ M^{-1} sec^{-1}, respectively. The corresponding values for poly[d(DAP-A-T)] were $5.6 \pm 2.4 \times 10^5$ and $2.2 \pm 0.6 \times 10^4$ M^{-1} sec^{-1}. For poly[d(DAP-A-T)], the total change in transmittance of the reaction, ΔT_{total}, increased when the nucleotide/antibiotic ratio, i.e., the concentration of binding sites, was increased. For example, at a nucleotide/actinomycin molar ratio of 18, ΔT_{total} for poly[d(DAP-A-T)] was 108 mV; at a ratio of 39, ΔT_{total} was 195 mV, which was very similar to values obtained at lower ratios with poly[d(DAP-T)] and DNA.

In 0.001 *M* potassium phosphate buffer at 36° (5.5° above the T_m), the kinetic behavior of the binding of actinomycin D to poly[d(DAP-A-T)] differs markedly from that observed at lower temperature (Table V). Under these conditions, ΔT_{total} was only 47 mV, indicating that at equilibrium less actinomycin is bound at 36° than at 25°. For comparison, it should be noted that the binding capacity at 25° of heat-denatured CT-DNA was lower than that of native DNA (ΔT_{total} of heat-denatured DNA was 120 mV at DNA/actinomycin molar ratios of 18 and 31, compared to 190 mV for native DNA); however, the reaction of actinomycin D with heat-denatured DNA at 25° is characterized by k_3, k_4, and k_5 values that are similar to those found with native DNA and the double-helical d(A-T)-like copolymers. This may be attributed to the renaturation that occurs when heat-denatured DNA is cooled. The absence of the two slowest reaction steps with poly[d(DAP-A-T)] above its melting temperature is the most striking difference between the kinetic parameters of actinomycin binding to denatured poly[d(DAP-A-T)] and those of binding to native poly[d(DAP-A-T)], poly[d(DAP-T)], DNA, and PM2 DNA, and to heat-denatured DNA at 25°. This difference cannot be attributed merely to a temperature effect on the rate processes since the five reaction steps for association of the antibiotic with DNA are observable at 36°. These results suggest that at least the two slowest steps are associated with an actinomycyl-pentapeptide-induced helix unwinding of the sugar–phosphate backbone. Furthermore, when poly[d(DAP-A-T)] is mixed above its T_m with actinomycin, intercalation in the normal sense between DAP-T and T-A base pairs cannot occur initially because the helical duplex in the uncomplexed polynucleotide has been disrupted by temperature. The first binding step thus probably does not represent intercalation of the phenoxazone group between base pairs in double-stranded regions. The observations that with DNA the entropy of activation of the first step is more negative than commonly encountered with bimolecular reactions and that the second-order rate constant is several orders of magnitude slower than diffusion controlled suggest that a considerable degree of overlap of the nonpolar surfaces of the phenoxazone ring and G-C or DAP-T base pairs may occur in this step; other interactions leading to restricted motional freedom of the reactants may also be involved.

Reaction steps designated by k_1 and k_2 are considerably faster in heat-denatured DNA than in native DNA. The step designated by k_1 was not detected in the binding of actinomycin D and actinomycin lactam to PM2 DNA (Table V). The k_1 step was observed, however, with a preparation of SV 40 DNA containing approximately 50% closed circular molecules. These results suggest that step 1 requires the presence of a relatively open conformation and is accompanied by large transmittance changes when the polynucleotide is partially disordered, as with heat-dentured DNA and poly[d(DAP-A-T)] above its T_m. Undetectably small transmittance changes result when the conformation is highly ordered, as in the superhelical PM2 DNA molecule. The value of k_2 is somewhat higher for binding of actinomycin D to the closed circular DNA than to the other DNAs, and k_3, k_4, and k_5 are approximately the same as those found with the open-chain DNAs.

As was observed for the binding of actinomycin lactam to DNA (Table II), the rate constants for steps 3, 4, and 5 are lower for the interaction of the lactam with PM2 DNA than for the corresponding reactions with the lactone (Table VI). This agrees with the suggestion based on data obtained with linear DNA that greater conformational restraints for accommodation of the pentapeptide rings into DNA may exist in the lactam than in the lactone. Rate constant k_2 is also lower for the PM2 DNA–lactam interaction than for the PM2 DNA–lactone interaction.

Table VI: Pseudo-First-Order Rate Constants and Transmittance Changes for Association of Actinomycin D and Actinomycin Lactum with PM2 DNA.[a]

Actinomycin	k_2	% ΔT_2	k_3	% ΔT_3	$10^2 k_4$	% ΔT_4	$10^3 k_5$	% ΔT_5
D	8.1 ± 0.5	22	0.55 ± 0.08	32	7.3 ± 4.0	28	7.9 ± 2.0	17
Lactam	2.1 ± 0.7	38	0.08 ± 0.01	14	1.5 ± 0.8	14	2.8 ± 0.3	34

[a] The ratio of DNA(P)/actinomycin was 18. Rate constants are reported in sec^{-1}.

Analysis of the interaction of actinomycin D with poly[d(G-C)] showed the presence of very fast and very slow processes whose rate constants were similar to those found for interaction with DNA and DAP-containing polynucleotides. The low degree of binding (ΔT_{total} was 19 mV at a nucleotide/actinomycin molar ratio of 16, and 36 mV at a molar ratio of 40) did not permit a full analysis of the kinetics of association, but the observation of slow rate processes in the actinomycin–poly[d(G-C)] association supports the conclusion that structural heterogeneity is not a prerequisite for a multiplicity of slow reactions.

Discussion

The kinetic analysis indicates that the maximum number of spectrophotometrically distinguishable forms of bound actinomycin that exist at equilibrium is five. Since the extent to which the reactions proceed to completion is not clear, the number of forms may be considerably less than five, i.e., many reactions may represent the conversion of one intermediate to the next intermediate in the course of attaining the most stable arrangement(s) of the interacting groups. If some of these partial reactions do not proceed to completion, the observed rate constant is actually a rate constant for approach to equilibrium and would include a component of the reverse rate constant.

Normalization of the bimolecular rate constants to the mole percent of potential binding sites (41 mol % for DNA, 10 mol % for poly[d($\overline{\text{DAP}}$-A-T)], and 100 mol % for poly[d($\overline{\text{DAP}}$-T)]) gives the following values for k_1: DNA, 9.2 ± 2.3 × 10^5; poly[d($\overline{\text{DAP}}$-A-T)], 56 ± 24 × 10^5; and poly[d($\overline{\text{DAP}}$-T)], 5.5 ± 2.5 × 10^5 M^{-1} sec^{-1}. For k_2, the values are: DNA, 10.8 ± 1.4 × 10^4; poly[d($\overline{\text{DAP}}$-A-T)], 22 ± 6 × 10^4; and poly[d($\overline{\text{DAP}}$-T)], 1.7 ± 0.3 × 10^4 M^{-1} sec^{-1}. Similar values are obtained if the normalization is carried out according to the number of binding sites calculated at equilibrium from spectrophotometric titrations (Table I). The order observed for both steps is poly[d($\overline{\text{DAP}}$-A-T)] > DNA > poly[d($\overline{\text{DAP}}$-T)]. Since the A-T base pair has one less hydrogen bond than the G-C or $\overline{\text{DAP}}$-T base pair and may be less extensively stacked, one would expect d(A-T) regions to be weaker than d(G-C) and d($\overline{\text{DAP}}$-T) regions. The observed trend in k_1 and k_2 thus follows the order of ease of disruption of the helical structure expected for these polynucleotides; in fact, we found the T_m values of poly[d($\overline{\text{DAP}}$-A-T)] and DNA in 0.001 M potassium phosphate buffer to be 30.5 and 50.5°, respectively. We therefore propose that some disruption of the double-helical conformation takes place in the initial two steps of actinomycin binding. It is of interest to note that although the normalized apparent bimolecular rate constants for steps 1 and 2 are lower for the d($\overline{\text{DAP}}$-T) copolymer than for poly[d($\overline{\text{DAP}}$-A-T)] and DNA, poly[d($\overline{\text{DAP}}$-T)] binds actinomycin more tightly at equilibrium (Table I). This is not a contradiction because, according to transition-state theory, rates are determined by the difference in free energy of the reactants and their transition states; equilibrium constants, however, are determined by the difference in standard free energy of the reactants and the complexes.

Two steps were detected in the binding of actinomine to DNA in 0.01 M potassium phosphate buffer (Table II). The rate constants of these reactions are lower than the rate constant of the one DNA concentration-dependent step observed by Müller and Crothers (1968) in buffer containing 0.38 M Na^+. Although we did not investigate the detailed concentration dependence of the pseudo-first-order steps k_1 and k_2 shown in Table II, from studies at two DNA concentrations we estimate the apparent second-order rate constants of these reaction processes to be approximately 3.0 × 10^6 and 1.9 × 10^5 M^{-1} sec^{-1}. These are not normalized to G-C content because absorption studies show that actinomine binds both to poly[d(A-T)] and to DNA (L. Blau and R. Bittman, unpublished results). The apparent second-order rate constants obtained for steps 1 and 2 of the actinomine–DNA interaction differ by a factor of about 3 or less from the normalized second-order rate constants calculated for the actinomycin D–DNA interaction. If the bulky peptide groups of actinomycin were involved in the first two reaction processes, the binding of actinomycin at G-C base pairs would be expected to be considerably slower than that of actinomine. If the initial rate processes in the interaction of DNA with actinomycin D are assumed to be similar mechanistically to the rate processes observed for DNA–actinomine interaction, the specificity of actinomycin for nucleotide sequences of defined secondary structure is expected to be dependent on interactions involving the cyclic peptides in subsequent steps. The observation that actinomycin D and actinomycin lactam have similar rate constants for steps 1 and 2, but not for steps 3, 4, and 5 (Table II), supports this suggestion. It is concluded from the results obtained with actinomine and actinomycin lactam that steps 1 and 2 represent principally interaction between the phenoxazone ring with the DNA binding site, with little contribution from the peptides.

Subsequent to the fastest reaction step, two rate processes (having rate constants k_2 and k_3) were detected in the complexation of actinomycin D with denatured poly[d($\overline{\text{DAP}}$-A-T)] (Table V). With this polynucleotide below the T_m, and with DNA, heat-denatured DNA, poly[d($\overline{\text{DAP}}$-T)], and the superhelical DNA, the conversion of the intermediate stages of bound actinomycin to the fully bound form(s) gives rise to two additional rate processes (characterized by rate constants k_4 and k_5). Because the sugar–phosphate backbone of native DNA is less readily distorted than that of a thermally disordered polynucleotide, we suggest that steps 3, 4, and 5 may represent the slow local unwinding, distortion, or conformational change(s) induced in the polynucleotide backbone when the double-stranded DNA interacts with the pentapeptide of actinomycin in the late stages of complex formation. Other investigators have proposed that complex formation re-

quires a change in deoxyribose puckering, especially of the deoxyguanosine residues (Sobell, 1973), and the unwinding of the helix in supercoiled DNA (Waring, 1970). Additional support for the proposal that the slow reactions require the existence of a double-stranded Watson-Crick structure comes from the experiments we performed with poly(dI), which is not a double-stranded polynucleotide. No slow association reactions were observed with poly(dI), both in the presence and absence of 0.03 *M* Mg^{2+}. The kinetics of the fast processes were also different from those observed with DNA. The type of interaction is probably quite different from that with natural and synthetic DNAs having Watson-Crick structures. Furthermore, only a very small amount of actinomycin binds to poly(dI) and its spectrum differs from that of actinomycin in the presence of DNA (Wells and Larson, 1970).

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