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Equilibrium Studies of Ethidium–Polynucleotide Interactions†

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ABSTRACT: We report equilibrium dialysis studies of the binding of ethidium to a variety of double-helical synthetic polynucleotides containing A·U (or T) and I·C base pairs. The results are interpreted in terms of the neighbor exclusion model of drug binding, with allowance both for cooperativity of binding and for a structural switch of the helix to a different form which binds the drug more effectively. Both DNA and the alternating copolymers examined [poly[d(A-T)] and poly[d(I-C)]] showed high affinity (10^4–10^5 M^{-1}) in 1 M salt. Homopolynucleotides showed a more complicated pattern of affinities: poly(rA)·poly(rU), poly(rA)·poly(dT), and poly(dA)·poly(rU) showed high affinity, whereas poly(dA)·poly(dT), poly(rI)·poly(rC), and poly(dI)·poly(dC) showed low affinity ($\leq 10^3$ M^{-1}). The neighbor exclusion range was inferred to be two base pairs for DNA or B family helices and three for RNA or A family helices. Generally, polynucleotides showed some cooperativity in their ethidium binding. The data reveal a switch of poly[d(I-C)] to a form able to bind ethidium more effectively.

The cationic dye ethidium has found exceptionally wide use in biochemical and physical studies of nucleic acids, ranging from utilization as a fluorescent stain to crystallographic studies of its complex with oligonucleotides (Tsai et al., 1975). A problem of long-standing interest has been the extent and origin of specificity in the intercalation of ethidium into double-helical nucleic acids. Little dependence of the binding affinity on overall base composition is found (Waring, 1965; Le Pecq & Paoletti, 1967; Müller & Crothers, 1975), but the work of Krugh and his colleagues has established a definite preference for binding to pyrimidine-(3′–5′)-purine sequences compared to purine-(3′–5′)-pyrimidine in both ribo- and deoxyribooligonucleotides (Krugh et al., 1975; Krugh & Reinhardt, 1975; Kastrup et al., 1978). A similar conclusion was reached by Patel & Canuel (1976).

A general description of drug–nucleic acid binding equilibria requires not only specification of the binding constant of the drug to an isolated binding site but also a description of how the bound drug molecules interact. It has long been recognized that intercalative binding saturates well before occupation of all the spaces between the base pairs, a phenomenon which has been explained by exclusion of drug molecules from empty sites which are adjacent to a bound drug (Cairns, 1962; Crothers, 1968). Intercalative binding of ethidium to deoxyribonucleic acid (DNA)[1] saturates at one drug per two base pairs, and the complete binding isotherm fits with high accuracy to that calculated by using the neighbor exclusion model (Bauer & Vinograd, 1970; Bresloff & Crothers, 1975). The alteration of sugar pucker found in the crystal of ethidium with double-helical dinucleotides has been invoked to explain the

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[1] Abbreviations used: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; EDTA, ethylenediaminetetraacetic acid; EB, ethidium bromide; Tris, tris(hydroxymethyl)aminomethane.

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every other base pair binding rule (Tsai et al., 1975).

Drug binding affinity and neighbor exclusion interactions can be influenced not only by base sequence but also by double-helix structure. In this paper we report equilibrium dialysis binding studies of the interaction of ethidium with a series of double-helical polynucleotides. We find a wide range of binding affinities, and in some cases we observe moderate cooperative effects in binding. The neighbor exclusion interaction limits binding to one drug every two or three base pairs, depending on the helix form. In two cases ethidium induces a switch in helix form, as reflected by an abrupt alteration in binding properties. A more detailed description of the results is given by Bresloff (1974).

The double-helical nucleic acids which we studied include some homopolynucleotides in which the 1:1 mixture is capable of disproportionating into triple helix and a polypurine strand. Of these, Riley et al. (1966) showed that poly(rA)·poly(rU), poly(dA)·poly(dT), and poly(rA)·poly(dT) exist as the double helix in high-salt solution and room temperature. Similarly, poly(rI)·poly(rC) and poly(dI)·poly(dC) at high salt were identified as the double helix by Chamberlin & Patterson (1965) and Inman & Baldwin (1964), respectively. A 1:1 mixture of poly(dA) + poly(rU) is converted from triple to double helix by ethidium binding (Lehrman & Crothers, 1977).

We express one word of caution for quantitative comparison of our results with other data: all our experiments were done on samples containing 1 M NO_3^- (an anion chosen because of its less deleterious effects than Cl^- on metal electrodes in subsequent temperature-jump studies; Bresloff, 1974). Binding constants are roughly 5 times smaller in nitrate than chloride (N. Dattagupta, private communication), presumably because of formation of a complex between ethidium and the planar nitrate anion.

Materials and Methods

Ethidium bromide was purchased from Boots Pure Drug Co. Ltd., Nottingham, England (batch no. DR 1731) and Calbiochem, Los Angeles, CA (lot 100301 B grade). Its purity was monitored by NMR spectroscopy and paper chromatography. Except when noted, experiments were performed in a 1 M Na^+ buffer (buffer II) with nitrate as the anion, formed by addition of an aqueous solution of 4.932 M $NaNO_3$ to buffer I (see below) in a volume of 1:4, pH 6.6. For details, refer to Bresloff & Crothers (1975).

Calf thymus DNA (type I) was purchased from Sigma Chemical Co., St. Louis, MO; *Micrococcus luteus* DNA, poly(rA), poly(rU), and poly[d(A-T)]·poly[d(A-T)] were purchased from Miles Laboratories, Elkhart, IN; poly[d(I-C)]·poly[d(I-C)], poly(dI)·poly(dC), poly[d(A-T)]·poly[d(A-T)], poly(dA)·poly(dT), poly(rA)·poly(dT), poly(dA)·poly(rU), poly(rI), and poly(rC) were purchased from P-L Biochemicals, Milwaukee, WI. DNA and polyribonucleotides were sonicated and purified by phenol extraction as previously described (Bresloff & Crothers, 1975). All other nucleic acids were directly dissolved in a low-salt buffer (buffer I: 0.006 M Na_2HPO_4, 0.002 M NaH_2PO_4, 0.001 M Na_2EDTA, and 0.001 M sodium cacodylate, pH 7.2) and extensively dialyzed.

Nucleic acid concentrations were determined spectrophotometrically in buffer I, except for poly(dI)·poly(dC) and poly(dA) + poly(rU) where the ionic strength was increased to 1 M Na^+ in chloride because of the low thermal stability of these nucleic acids. The following molar extinction coefficients per centimeter of solution ($\times 10^{-3}$) were used: poly(rA), ϵ_{257} = 10.5, poly(rU), ϵ_{261} = 9.5, and poly(rA)·poly(rU), ϵ_{260} = 7.14 (Krakauer, 1969); poly[d(A-T)]·poly[d(A-T)], ϵ_{260} = 6.65, and *M. luteus* DNA, ϵ_{260} = 6.965 (Felsenfeld & Hirschman, 1965); poly(rI), ϵ_{248} = 10.0, poly(rC), ϵ_{268} = 6.2, and poly(rI)·poly(rC), ϵ_{260} = 5.0 (Chamberlin & Patterson, 1965); poly(dI)·poly(dC), ϵ_{245} = 5.3 (Inman & Baldwin, 1964); poly(dA)·poly(dT), ϵ_{259} = 6.0, poly(rA)·poly(dT), ϵ_{257} = 6.9, and poly(dA) + poly(rU), ϵ_{257} = 6.5 (Riley et al., 1966); poly[d(I-C)]·poly[d(I-C)], ϵ_{251} = 6.9 (Grant et al., 1968); calf thymus DNA, ϵ_{260} = 6.55 (average of several literature values).

These nucleic acids were characterized by their melting transitions (Bresloff, 1974); agreement between our results and the literature values was, in general, quite good. The synthetic DNAs were further characterized by their circular dichroism spectra, since this technique reveals large differences in optical activity between them. Excellent agreement was found between the peak, trough, and crossover wavelengths of our spectra with the results of Wells et al. (1970), although ratios of the various peak to trough amplitudes did not always agree. Slight deviations in these physical measurements from reported results might to bue to differences in molecular weight of the nucleic acids or to the buffer systems used.

Binding iostherms in buffer II at 19.0 °C were determined by equilibrium dialysis with analysis by difference absorption spectroscopy. Dialysis was typically allowed to continue for 65 h at constant temperature in a standard dialysis bag. Measurement of the absorbance of the dialyzate gives the free dye concentration of the complex, and difference absorption at 19.0 °C, where the reference compartment of the spectrophotometer contains the dialyzate of free dye while the sample compartment contains the complex of bound and free dye, yields directly the spectrum of the bound dye at a particular r value. When quantities of nucleic acid were plentiful [i.e., calf thymus DNA, *M. luteus* DNA, poly(rA)·poly(rU), and poly(rI)·poly(rC)] two solutions were dialyzed at each r value, with nucleic acid concentrations giving maximum visible band absorbancies of the bound dye between 0.1–0.2 and 1.0–2.0. In general, the two r values obtained agreed to within 3% except at high r where solutions with low nucleic acid concentration ($\sim 1 \times 10^{-4}$ M base pairs) gave anomalously lower r values. This may be due to adhesion of nucleic acid to the membrane, facilitated by the presence of bound EB. Although bound-dye spectra were found to be essentially independent of r, anomalously high absorbance readings occurred at the lowest extents of binding in the low-wavelength region, e.g., 360 nm. This was shown to be due to scattering from the concentrated polynucleotide solution required at these low r values by observing the scattering from concentrated nucleic acid solutions free of dye. Bound dye spectra were taken by using 10-mm water-thermostated cells (Hellma Cells, Inc., Jamaica, NY) or 1-mm cells when total absorbances exceeded $\sim$2 OD. Free dye spectra were meausred with 1-, 10-, or 100-mm path length cells. All work was done at 19.0 °C. All measurements were performed on a Cary 14 recording spectrophotometer, using the expanded 0–0.2 slide-wire whenever possible.

The extinction coefficient of monomeric free EB was found to be 5850 $(M \cdot cm)^{-1}$ at 480 nm in buffer I at 19.0 °C and 5820 in buffer II under the same conditions (Bresloff & Crothers, 1975). The extinction coefficient of bound EB was obtained by the following procedure: a dye–nucleic acid complex was dialyzed to equilibrium, and the absorption spectrum of the bound dye at the resulting r value was obtained. Addition of an organic solvent which prevents dye binding to the nucleic acid to both the complex and dialyzate, followed by difference absorption, allows for direct determination of the concentration of bound dye as long as the extinction coefficient of the free

dye in the buffer–organic solvent system is known. The extinction coefficient of the bound dye at all wavelengths is thus directly determined.

In practice, ethanol was added to complexes in buffer II in the ratio of 2:1 (v/v). Solutions were allowed to sit overnight and then centrifuged, since addition of ethanol results in precipitation of the nucleic acid. That addition of ethanol can be used to determine the concentration of bound dye was deduced by showing that two solutions having identical total concentrations of EB but with one containing calf thymus DNA and the other not, give identical absorbancies after addition of ethanol. The apparent extinction coefficient at λ_{max} = 519 nm for EB in ethanol–buffer II (2:1 v/v) at 19.0 °C was found to be 6430. (Including the volume change upon mixing gives ϵ_{max} = 6230.) The extinction coefficient of the bound dye at λ_{max} for all the nucleic acids tested, at 19.0 °C, was found to be 4150 ± 30 (±1 standard deviation) by utilizing this system. These polymers with their respective r values are as follows: calf thymus DNA (0.081 and 0.38); *M. luteus* DNA (0.092 and 0.30); high molecular weight calf thymus DNA (0.080); poly(rA)·poly(rU) (0.12 and 0.32); poly(rI)·poly(rC) (0.086); poly[d(A-T)]·poly[d(AT)] (0.14 and 0.47); poly(rA)·poly(dT) (0.291); poly(dA)·poly(rU) (0.284). From this it has been assumed that ϵ_b at λ_{max} for all the double-helical nucleic acids used in this study at all r values is equal to 4150 at 19.0 °C.

Results

Aggregation of Ethidium. Ethidium binding to nucleic acids is weakened by the high salt concentrations used in our experiments (Waring, 1965; Le Pecq & Paoletti, 1967), so relatively high dye concentrations are required to approach saturation of binding. Hence, nonideality of the dye solution cannot be ignored. We assume that nonideality results from aggregation of the drug, which, at low degrees of association can be adequately approximated by a dimerization equilibrium:

$$2 \text{ monomers} \overset{K_d}{\rightleftharpoons} \text{dimer}$$

We evaluated the dimerization equilibrium constant K_d spectrophotometrically by using the equation derived by Bresloff (1974)

$$\frac{1}{\epsilon_m - \epsilon_{ap}} = \frac{2}{2\epsilon_m - \epsilon_d} + \frac{1}{C_m}\left[\frac{1}{K_d(2\epsilon_m - \epsilon_d)}\right] \quad (1)$$

in which ϵ_m and ϵ_d are the molar extinction coefficients of monomer and dimer, respectively, and ϵ_{ap} is the apparent extinction coefficient, related to the absorbance A in a 1-cm path by

$$A = \epsilon_{ap}C_t = \epsilon_m C_m + \epsilon_d C_d$$

where the total molar concentration C_t is the sum of monomer and dimer contributions, or $C_m + 2C_d$.

An iterative procedure (Bresloff, 1974) was used to plot the left side of eq 1 against C_m in order to obtain K_d and ϵ_d from the slope, $1/[K_d(2\epsilon_m - \epsilon_d)]$, and intercept, $2/(2\epsilon_m - \epsilon_d)$. Initially, it was assumed that $C_m = C_t$, and K_d was calculated, from which a better estimate of $C_m < C_t$ could be obtained. This procedure was repeated until self-consistent results were obtained in which the value of K_d used to calculate C_m was found to equal the ratio of the intercept to twice the slope. A related procedure has been used by Martin (1980) for daunorubicin dimerization.

We found values of $K_d = 2.9 \times 10^2$ M^{-1} in 1 M $NaNO_3$ at 19 °C and $K_d = 2.7 \times 10^2$ M^{-1} in 1 M NaCl at 19 °C. Knowledge of the dimerization constant and the total free dye concentration in a dialysis equilibrium experiment then allows us to calculate the concentration of monomeric free dye C_m necessary to produce a given binding occupancy r. This ensures that the standard state for all binding equilibrium constants remains the monomeric free dye. At the highest C_t values utilized in binding isotherms reported here, the monomer was ~76% of the total free dye concentration.

Interpretation of Binding Isotherms. We used both the Scatchard (1949) and neighbor exclusion (Crothers, 1968; Zasedatelev et al., 1971; McGhee & von Hippel, 1974) equations to analyze the data. The Scatchard formulation assumes independent identical binding sites, B_{ap} per base pair, of binding affinity K_{ap}, and gives for the isotherm

$$r/C_m = K_{ap}(B_{ap} - r) \quad (2)$$

in which r is the number of drug molecules bound per base pair. This linear equation fits only a limited range of the binding isotherm.

In order to fit the data by using the neighbor exclusion model, we found it necessary to allow for cooperative interaction between bound dye molecules separated by the minimum distance, as was also the case in our earlier study on actinomycin analogues (Müller & Crothers, 1968). We let $K(0)$ be the intrinsic binding constant for a dye molecule isolated on the DNA; $K\tau$ is the binding constant when a drug binds the minimum distance away from one other drug molecule. McGhee & von Hippel (1974) give equations for calculating binding isotherms for this model. Our calculations actually utilized the equivalent parametric equations derived (Bresloff, 1974) by use of the method of sequence-generating functions (Lifson, 1964)

$$r = \frac{(\tau - 1)y^2 + (1 - 2\tau)y + \tau}{(n\tau - n)y^2 + (1 + n - 2n\tau)y + n\tau} \quad (3a)$$

$$K(0)C_m = \frac{ry}{(1 - \tau)y^{n+1} + \tau y^n} \quad (3b)$$

where y is a number between 0 and 1 and n is the neighbor exclusion range, equal to the minimum number of base pairs which must intervene between successive intercalated ethidium residues. (There are $n - 1$ empty binding sites between adjacent dyes at saturation.) The more complicated binding equilibria which arise when drug binding induces a change in nucleic acid structure are considered later.

Isotherms Which Fit the Neighbor Exclusion Model. Figures 1–4, and Table I, present the properties of binding isotherms which we could fit by using the neighbor exclusion model, with nearest-neighbor cooperativity where needed. The binding curve for DNA from two sources (Figure 1) is apparently noncooperative and shows the characteristic curvature of the neighbor exclusion isotherm. At the other extreme is the isotherm for the alternating copolymer poly[d(A-T)], showing the opposite curvature, which is accounted for in the calculation by adjusting τ to 2.3.

Of particular note in this series is the observation that the neighbor exclusion range n is 2 for DNA and the deoxypolynucleotide poly(dA-dT), whereas n is 3 for the double-helical polyribonucleotides. This suggests the possible general rule that (for ethidium binding) the B family of helices has n = 2 whereas the A family has n = 3. This rule is consistent with our observations on other polymers.

Weakly Binding Polymers. Figure 5 shows isotherms for two deoxy(polypurine·polypyrimidine) double helices which bind ethidium only weakly. We found nearly flat isotherms,

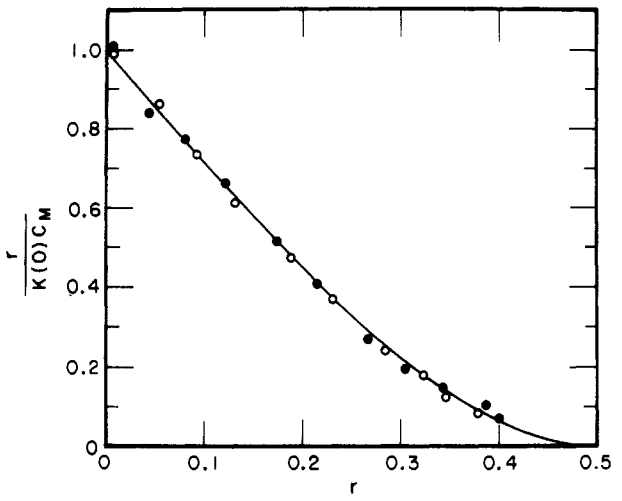

FIGURE 1: Equilibrium dialysis isotherm for ethidium binding to *M. luteus* (○) and calf thymus (●) DNA, compared with the isotherm calculated by using the neighbor exclusion model, $n = 2$ and $\tau = 1$. Isotherms were normalized by division by $K(0)$, which is the limiting value of r (in base pairs) divided by monomer concentration C_m as r goes to 0. Values of $K(0)$ are given in Table I. Experimental conditions: buffer II; 19.0 °C.

Table I: Binding Isotherm Parameters for Ethidium and Nucleic Acids[a]

	Scatchard analysis		neighbor exclusion analysis		
nucleic acid	K_{ap} (M^{-1})	B_{ap}	$K(0)$ (M^{-1})	n	τ
calf thymus DNA	5.1×10^4	0.36	1.83×10^4	2	1.0
M. luteus DNA	6.0×10^4	0.36	2.16×10^4	2	1.0
poly(rA)·poly(rU)	1.1×10^5	0.30	3.1×10^4	3	2.3
poly(dA)·poly(dT)			$\sim 1 \times 10^3$		
poly[d(A-T)]·poly[d(A-T)]	5.3×10^4	0.59	2.9×10^4	2	2.3
poly(rI)·poly(rC)	2.0×10^3	0.30	6.0×10^2	3	1.9
poly(dI)·poly(dC)			$\sim 1 \times 10^3$		
poly[d(I-C)]·poly[d(I-C)]					
form 1			2.6×10^4	2-3	1
form 2			1.5×10^4	2	3
poly(rA)·poly(dT)	5.9×10^5	0.20	1.1×10^5	3	1.0
poly(dA) + poly(rU)					
triple helix (1)			1.6×10^4	3	1
double helix (2)			1.4×10^4	3	2.5

[a] 19 °C in 1 M $NaNO_3$. Nitrate anion produces about 5 times lower ethidium binding constants than chloride at the same 1 M concentration (N. Dattagupta, private communication).

from which it was not possible to obtain accurate binding parameters, given the small amount of polynucleotide we had available. Notice (Table I) that poly(dA)·poly(dT) binds ~30 times more weakly [comparing $K(0)$ values] than poly(rA)·poly(rU), but poly(dI)·poly(dC) and poly(rI)·poly(rC) are about equally weak in binding affinity.

Induced Conformational Changes. In two cases we studied, ethidium causes an apparent change in helix structure to a new form able to bind ethidium more effectively. A well-studied example of such a phenomenon is the switch of poly[d(G-C)] between helix forms, as discovered by Pohl & Jovin (1972). Recent structural work (Wang et al., 1979; Arnott et al., 1980) indicates that the weakly binding helical form is a left-handed helix.

The shape of the ethidium binding isotherm of poly[d(I-C)] shown in Figure 6 does not fit any simple model for cooperative or anticooperative (neighbor exclusion) binding to an unchanging lattice. Rather, the molecular appears to switch between two different binding behaviors, from which we infer an alteration in molecular structure. However, the structural

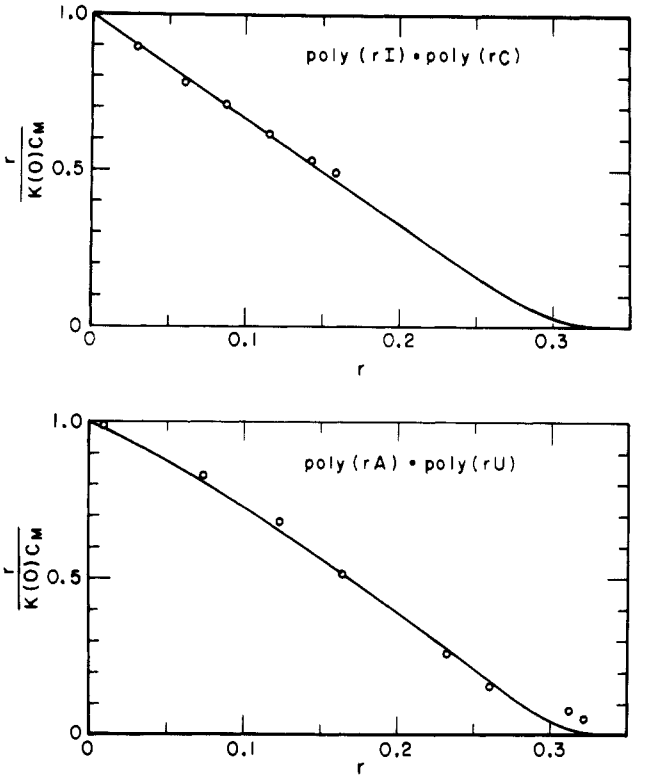

FIGURE 2: Experimental and calculated neighbor binding isotherms: (a) for poly(rI)·poly(rC), $n = 3$, $\tau = 1.9$; (b) for poly(rA)·poly(rU), $n = 3$, $\tau = 2.3$. For further details see the caption to Figure 1.

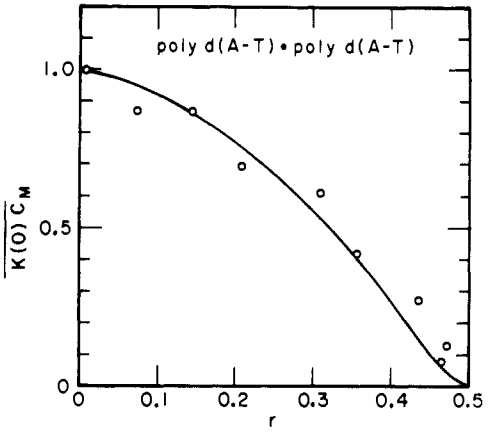

FIGURE 3: Experimental and calculated neighbor exclusion binding isotherm for polyd(A-T)·polyd(A-T), $n = 2$, $\tau = 2.3$.

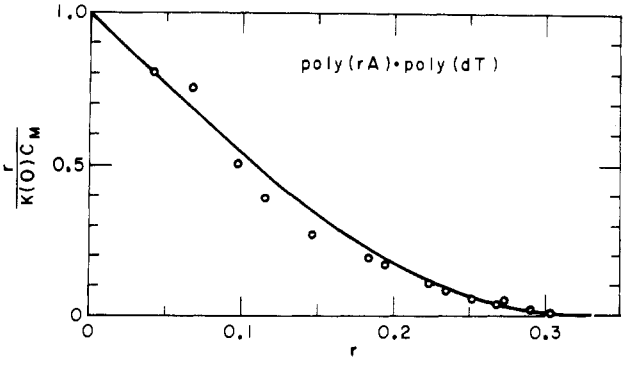

FIGURE 4: Experimental and calculated neighbor exclusion binding isotherms for poly(rA)·poly(dT), $n = 3$, $\tau = 1.0$.

basis for the conformational switch remains unknown in this case.

We have analyzed the binding equilibria shown in Figure 6 by using the theory for induced allosteric changes in DNA

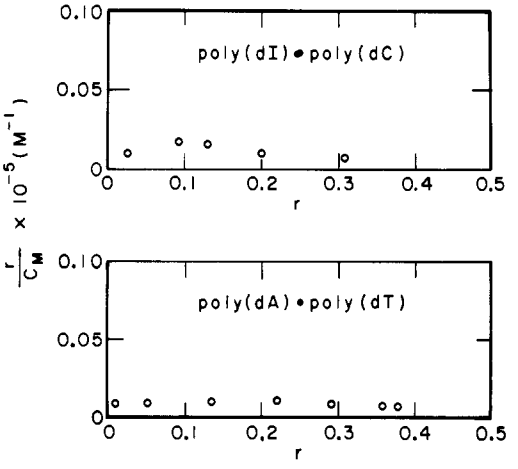

FIGURE 5: Equilibrium binding isotherms for ethidium with (a) poly(dI)·poly(dC) and (b) poly(dA)·poly(dT). Buffer II, 19 °C.

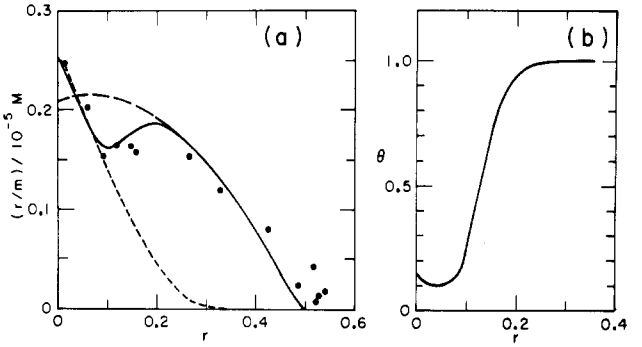

FIGURE 6: (a) Binding data for double-helical poly[d(I-C)], buffer II, 19 °C, compared with theoretical curves which allow for a switch between two helical forms and hence between two binding isotherms. The parameters for form 1 (---) and form 2 (———) are collected in Table I: the solid line shows the calculated binding isotherm which switches between the two extremes. The parameter $s = 0.98$ and $\sigma = 0.01$. (b) Calculated fraction of bases in form 2 (θ), indicating the structural transition which is inferred from the binding data.

which we presented recently (Dattagupta et al., 1981). We found for the present problem that it was necessary to allow for cooperative binding of the drug to the individual helix forms. Briefly, the model allows two structures, 1 and 2, for the helix. Each is characterized by intrinsic binding constant K, neighbor exclusion range n, and cooperativity parameter τ, in which $K\tau$ is the binding constant for a drug bound the minimum distance from one other bound drug molecule. In addition, the equilibrium constant per base pair for converting structure 1 to 2 is s, and the equilibrium constant for nucleating helix form 2 in the middle of form 1 is $\sigma^2 s$ (Dattagupta et al., 1981).

A minor extension of the method of sequence-generating functions allows one to calculate binding isotherms for this model. The series F is a summation of the statistical weighting factors of drug-free regions of the molecule

$$F_1 = \sum_{j=1}^{\infty} y^j$$

$$F_2 = \sum_{j=1}^{\infty} (ys)^j$$

where factory y is included for each base pair, for reasons described below. The series summation begins with 1 because by definition a drug-free region of the molecule must include at least one base pair which is not part of an occupied site. Similarly, the series B represent regions in which bound drugs,

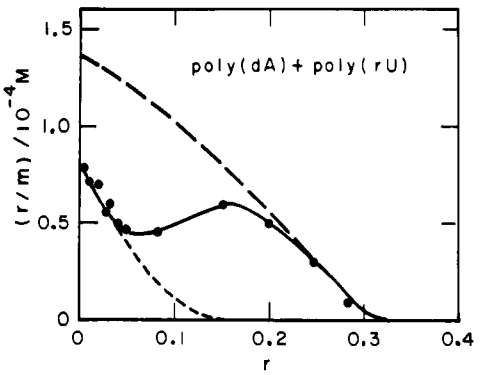

FIGURE 7: Ethidium binding to the 1:1 mixture of poly(rA) + poly(rU), showing the binding isotherms inferred for the triple helix (---) and double helix (———). Buffer II, 19 °C. The solid line shows the calculated binding, allowing for a switch between the two forms. In addition to the parameters collected in Table I, the curves were calculated with $s = 0.89$, $\sigma = 0.025$.

one through infinity in number, are separated by the minimum distance, with n base pairs per drug

$$B_1 = K_1 a y^{n_1} + K_1^2 \tau_1 a^2 y^{2n_1} + \ldots = \frac{K_1 a y^{n_1}}{1 - K_1 \tau_1 a y^{n_1}}$$

$$B_2 = K_2 a (ys)^{n_2} + K_2^2 \tau_2 a^2 (ys)^{2n_2} + \ldots = \frac{K_2 a (ys)^{n_2}}{1 - K_2 \tau_2 a (ys)^{n_2}}$$

in which a is the drug activity (taken equal to its free monomer concentration in our experiments). The sum of statistical weights of all possible sequences of bound and free region can be generated by repeated multiplication of the matrix $\mathbf{M}$ by

$$\mathbf{M} = \begin{pmatrix} 0 & B_1 & \sigma F_2 & \sigma B_2 \\ F_1 & 0 & \sigma F_2 & \sigma B_2 \\ \sigma F_1 & \sigma B_1 & 0 & B_2 \\ \sigma F_1 & \sigma B_1 & F_2 & 0 \end{pmatrix}$$

a unit vector. As described previously (Dattagupta et al., 1981), the parameter y is adjusted so that the largest eigenvalue of $\mathbf{M}$ is unity, ensuring that the partition function for the system is on its circle of convergence, and the average molecular length is infinity. Average properties are calculated by numerical differentiation.

Figure 6 shows a binding isotherm calculated to fit the poly[d(I-C)] data by using this theory. The dotted lines show the binding isotherms expected for the two forms when isolated. The switch of the binding behavior from one to the other (solid line) occurs at intermediate values of r. We note from Figure 6 and the parameters collected in Table I that the intrinsic binding constant K_2 is actually weaker than K_1 (the intrinsic binding constant is equal to the intercept of the isotherm on the r/m axis). The switch to form 2 appears to occur because of the possibility for cooperative binding of the drug to form 2, with $\tau_2 = 3$. Thus, at large values of r, the net affinity of form 2 is larger than that of form 1, and the relative stabilities of the two forms are inverted. Figure 6 also shows a calculated curve for θ, the fraction of base pairs which are in form 2. The steepness of this transition is controlled primarily by the parameter σ, with some contribution from τ_2. The neighbor exclusion range n_1 was taken equal to 3 for the calculation in Figure 6, but the data are not sufficiently precise

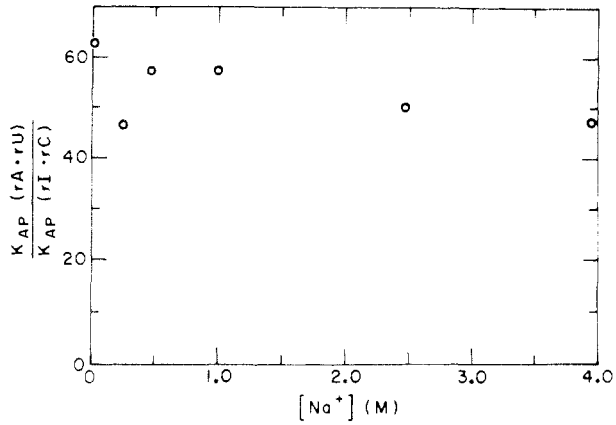

FIGURE 8: Ratio of the apparent binding constant of EB–poly(rA)·poly(rU) to EB–poly(rI)·poly(rC) as a function of ionic strength. The Na^+ concentration was varied by addition of appropriate volumes of an aqueous solution of 4.932 M $NaNO_3$ to buffer I. The pH progressively shifted from 7.2 in buffer I to 6.1 in 3.95 M Na^+. The experiment was performed at 19.0 °C.

to exclude the value 2. The cluster of data points at $r > 0.5$ cannot be accommodated to an integral value of n_2; it is possible that there is a small error (5–10%) in the actual drug to nucleic acid ratio in that experiment or that there is another weakly bound form of the drug.

Figure 7 shows the same theory applied to the triple helix → double helix switch of the poly(dA) + poly(rU) system which we documented previously (Lehrman & Crothers, 1977). In this case the statistical model is only approximate, since it treats the mixture of triple helix plus single strand of poly(rU) as a homogeneous lattice. The result is a calculated neighbor exclusion range of 6, which we correct to 3 in Table I because only the poly(dA) strand complexed with two poly(U)'s is assumed able to bind ethidium strongly. Similarly, the value of K_1 taken for the calculation in Figure 7 is corrected by multiplying by 2 in Table I to account for the same effect. Again in this case, the switch between two helix forms is apparent. Comparison of values in Table I and the isotherm in Figure 7 shows that the switch to double helix occurs not because of a difference in intrinsic binding affinities, but because the double helix offers twice as many binding sites as the triple helix plus single strand, and also because of the possibility for cooperative binding ($\tau_2 = 2.5$) to the double helix.

Comparison of Salt Dependence of EB Binding between Poly(rA)·Poly(rU) and Poly(rI)·Poly(rC). The binding isotherms of EB to the two synthetic RNAs poly(rA)·poly(rU) and poly(rI)·poly(rC) reveal that while the extent of neighbor exclusion is the same for both and the degree of cooperativity is similar, the intrinsic binding constant to an isolated site differs by over a factor of 50, being stronger for poly(rA)·poly(rU). This surprising behavior was investigated as a function of ionic strength especially because of the conflicting results that have been previously reported: Le Pecq & Paoletti (1965) reported that in 0.2 M NaCl–0.2 M Tris-HCl, Scatchard plots to both these RNAs are identical while Waring (1965) found large differences in the binding in 0.04 M Tris-HCl.

The binding characteristics at each salt concentration were determined by dialyzing, to the same free dye concentration, a solution containing each nucleic acid. By assuming that B_{ap}, which was determined at 1 M Na^+, remains independent of salt concentration, K_{ap} can be determined from the two binding isotherm points, and therefore K_{ap}[poly(rA)·poly(rU)]/[K_{ap}-[poly(rI)·poly(rC)]] can be calculated as a function of ionic strength. The results (Figure 8) indicate that the large difference in binding at 1 M Na^+ is also present at least between 0.02 and 4.0 M Na^+. Even if the magnitude of B_{ap} were dependent on salt concentration, it would still be found that the ratio of apparent binding constants is large as long as the ratio of B_{ap} values for the two nucleic acids is not much different from 1. Further, under buffer conditions similar to those used by Le Pecq & Paoletti (1965) (Tris-HCl–NaCl at 0.19:0.21 M, pH 7.4, and Tris-HCl–$NaNO_3$ at 0.19:0.21 M, pH 7.3), large values for the ratio of apparent binding constants are still found (48.4 and 49.5, respectively). From this we conclude that the intrinsic binding constant to an isolated binding site differs greatly with these nucleic acids over a wide range of ionic strength.

Table II: Wavelength of Maximum Visible Extinction Coefficient of EB Bound Dye Absorption Spectrum[a]

nucleic acid	λ_{max} (nm)
calf thymus DNA	521
high molecular weight calf thymus DNA	521
M. luteus DNA	521
poly(rA)·poly(rU)	518
poly(dA)·poly(dT)	523
poly[d(A-T)]·poly[d(A-T)]	520
poly(rI)·poly(rC)	518
poly(dI)·poly(dC)	520
poly[d(I-C)]·poly[d(I-C)]	522
poly(rA)·poly(dT)	520
poly(dA)·poly(rU)	520

[a] Buffer II; 19.0 °C.

Spectral Properties of Bound Dye. We found the extinction coefficient of the bound dye at λ_{max} to be independent of the nature of the polynucleotide used. However, there was some variation in λ_{max}, as reported in Table II. There seems to be no correlation of these λ_{max} values with binding affinity.

Discussion

Generalizations about the Intrinsic Binding Constant K(0). Our results collected in Table I show that all alternating-sequence double-helical polynucleotides, of which we have only polymers of the DNA type, bind ethidium tightly, with intrinsic binding constants ranging from 1.5×10^4 M^{-1} for form 2 of poly[d(I-C)] to 2.9×10^4 M^{-1} for poly[d(A-T)]. This is consistent with the observation of Krugh et al. (1975) that the deoxypyrimidine-(3′–5′)-purine sequence is a good ethidium binding site. However, purine–purine (or pyrimidine–pyrimidine) sites can provide even higher affinity, at least in helices of the A family, as judged by the intrinsic affinities of 3.1×10^4 M^{-1} for poly(A)·poly(U) and 1.1×10^5 M^{-1} for poly(rA)·poly(dT). A somewhat lower intrinsic affinity of 1.4×10^4 M^{-1} was found for the double helix of poly(dA)·poly(rU). In contrast, both the I·C-containing polymers poly(dI)·poly(dC) and poly(rI)·poly(rC) showed weak affinity, on the order of 10^3 M^{-1}.

All of this serves to emphasize, as originally stated by Krugh and his colleagues, that the preference for pyrimidine-(3′–5′)-purine binding sites is relative to purine-(3′–5′)-pyrimidine sites. Other strong sites exist, but whether they are strong or weak can depend on the helix structure. The intrinsic affinity for DNA lies within the range of values for the strong sites found in polynucleotides.

Generalizations about the Neighbor Exclusion Range n. With the possible exception of form 1 of poly[d(I-C)], all double helices of the DNA or B family appear to show a neighbor exclusion range of 2. However, helices presumably of the RNA or A family, including poly(rA)·poly(rU), poly-

(rI)·poly(rC), poly(rA)·poly(dT), and poly(dA)·poly(rU), all have a neighbor exclusion range of 3. Evidently some structural basis other than a simple alternating sugar pucker (Tsai et al., 1973) is required to explain the neighbor exclusion interaction in this case.

Generalizations about Cooperativity. Of the synthetic polynucleotides for which the binding isotherm is simple enough to allow good accuracy, only poly(rA)·poly(dT) apparently does not show cooperative binding ($\tau > 1$). In the other cases [poly(rA)·poly(rU), poly[d(A-T)], poly(rI)·poly(rC), poly[d(I-C)] form 2, and poly(dA)·poly(rU)], the presence of a bound dye the minimum distance away increases the affinity of a binding site by a factor of roughly 2–3. The structural basis for this effect is unknown, but it is well to keep in mind that a factor 3 in K corresponds to roughly RT in free energy, so the thermodynamic influence is small and it will be difficult to identify the source.

Why Is Ethidium Binding to DNA Not Cooperative? Given the complexity of ethidium interaction with polynucleotides, it is perhaps surprising that the interaction with DNA follows the simple neighbor exclusion model so precisely. It is possible that this apparent simplicity hides several counteracting influences. One expects some variability in the affinity of particular DNA sites, perhaps by a factor of 2–3 among the strong sites, because of sequence heterogeneity. This influence would produce anticooperative curvature throughout the isotherm, which would act in opposition to the cooperative curvature found with synthetic polynucleotides. We consider it possible that these contrary influences effectively cancel, producing a deceivingly simple equilibrium binding isotherm to DNA.

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