Proc. Nat. Acad. Sci. USA
Vol. 69, No. 12, pp. 3805–3809, December 1972

Ethidium Bromide as a Cooperative Effector of a DNA Structure

(poly(dG-dC)/allosterism/drug binding/kinetics/fluorescence/circular dichroism)

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Communicated by Manfred Eigen, October 6, 1972

ABSTRACT **A salt-induced cooperative conformational transition of a synthetic DNA, poly(dG-dC), is reversed by addition of ethidium bromide. Binding of the dye at high salt concentrations is highly cooperative. Circular dichroism spectra of the complex and the kinetic data support a model for this cooperative binding that is formally equivalent to the "allosteric" one proposed for oligomeric proteins by Monod *et al.* Thus, double-helical DNA of at least one defined sequence can undergo a cooperative conformational change in solution, with simple salts and drug molecules as antagonistic effectors. Such transitions may be involved in regulatory phenomena operating directly at the level of nucleic acid structure.**

The alternating synthetic polynucleotide, poly(dG-dC), changes at neutral pH in a highly cooperative way from one double-helical form to another when the salt concentration is increased above 2.5 M NaCl (1, 2). This conformational transition between the low-salt "*R*-form" and the high-salt "*L*-form" is characterized by an inversion of the circular dichroism spectrum and a redshift of the UV absorption spectrum. The transition after a shift of the salt concentration follows first-order kinetics and is rather slow, occurring at room temperature over several minutes (2).

To obtain information about the molecular properties of these two double-helical structures we have studied the binding of ethidium bromide at low- and high-salt concentrations. Ethidium bromide binds to double-stranded polynucleotides, presumably by intercalation (3, 4). The drug and related molecules also have been used in fluorescence staining of chromosomes (5, 6). Striking and reproducible banding patterns have been observed. The presence of bands of varying intensity in chromosomes may be due to not only different base composition (7) but also to different conformational states of the DNA. The R–L transition of poly(dG-dC) enables us to examine the interaction of drug molecules with different double-helical structures in solution.

MATERIALS AND METHODS

Poly(dG-dC) was synthesized with *Escherichia coli* DNA polymerase I and was characterized by nearest-neighbor analysis, the melting behavior, and molecular-weight determinations (2). The molar extinction coefficient at 255 nm is 7.1×10^3. Two samples were used that had sedimentation coefficients, $s_{20,w}$, of 1.8 S and 6.3 S in alkaline solution and 2.8 S and 6.5 S in neutral solution. These values correspond to double-stranded structures with about 20 and 400 nucleotides per strand, respectively (2, 8). The above samples, therefore, are referred to as $(dG\text{-}dC)_{\overline{10}}$ and $(dG\text{-}dC)_{\overline{200}}$. Ethidium bromide was a gift from Boots Pure Drug Co. and has a molar extinction coefficient $\epsilon_{480\text{ nm}} = 5450\ M^{-1}\ cm^{-1}$ (9). Solutions contained 20 mM Na-phosphate (pH 6.8) and 2 mM EDTA in addition to NaCl at the indicated concentrations. (In 4.4 M NaCl, the final pH was only 5.6, but parallel experiments gave identical results when the pH was kept at 7.0 at the different salt concentrations, indicating that there is no notable pH dependence in this range.)

Fluorescence measurements and continuous titrations were performed with a differential fluorimeter built by J. J. H. (to be published) and a Fica 55 spectrofluorimeter. Zeiss PMQ II and Cary 16 spectrophotometers were used for absorption measurements. Circular dichroism (CD) spectra were obtained on a Cary 60 spectropolarimeter with CD attachment. Cells were kept at 21° unless otherwise indicated.

Binding of ethidium bromide to the nucleic acid was followed by the increase in fluorescence at 590 nm with excitation at 510 nm for nucleic-acid concentrations of 0.4–10 μM. Measurements at poly(dG-dC) concentrations of 20–600 μM were performed by observation of the decrease of absorption at 470 nm ($\Delta\epsilon_{470} = -3200\ M^{-1}\ cm^{-1}$) or 284 nm ($\Delta\epsilon_{284} = -27200\ M^{-1}\ cm^{-1}$). Titrations were also done by the continuous linear addition of concentrated stock solutions of ethidium bromide to sample and reference cells with constant stirring. The differences in absorption or fluorescence were directly recorded as a function of time.

Equilibrium dialysis was not successful due to strong binding of the dye to the dialysis membrane. Other binding studies with $(dG\text{-}dC)_{\overline{200}}$, however, were performed in a Spinco Model E analytical centrifuge equipped with a scanning system at 20° and 40,000 rpm. The amounts of bound and free dye were followed at 305 or 511 nm. A few experiments involved gel filtration of $(dG\text{-}dC)_{\overline{200}}$ on a Biogel-P10 column in the presence of ethidium bromide.

We performed kinetic measurements at high-salt concentrations of processes related to conformational changes in the DNA after abruptly stopping the continuous addition of concentrated ethidium bromide solution or after adding and rapidly mixing small amounts of polymer, ethidium bromide, or buffer directly in the cell. A temperature-jump method with fluorescence detection was used for relaxation kinetic measurements of the binding process.

RESULTS

Binding Data. The interaction of ethidium bromide with polynucleotides can be observed by different optical methods

Abbreviation: Ethidium bromide, 3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide.

(3, 4). Fig. 1 shows the changes in the optical properties of the dye upon binding to poly(dG-dC).

There are hypochromic and bathochromic shifts in the absorption spectrum (Fig. 1*a*) similar to those reported for natural DNA. There are well-defined isosbestic points near 299, 318, 390, and 511 nm, consistent with a limited number of bound species. The absorption maximum in the visible shifts from 480 nm to about 520 nm. There are also dramatic changes in the fluorescence properties of ethidium bromide upon binding to poly(dG-dC) (Fig. 1*b*). The quantum yield

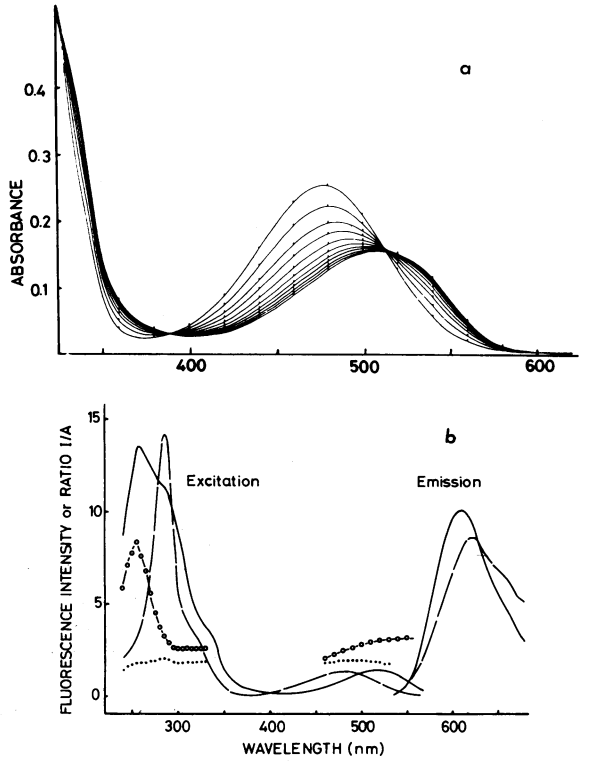


FIG. 1. Spectral evidence for binding of ethidium bromide to poly(dG-dC). (*a*) Recorded visible absorption spectra of ethidium bromide at $(dG\text{-}dC)_{\overline{200}}$ concentrations between 0 and 490 μM in 1.0 M NaCl. Equal amounts of a 5.4 mM $(dG\text{-}dC)_{\overline{200}}$ stock solution were added to a 1-cm cell. (The small marks on the spectra are produced by an electronic wavelength marker.) (*b*) Corrected fluorescence excitation and emission spectra of ethidium bromide free (- - -) and bound to poly(dG-dC) (——). The ratio of the excitation (I) and absorption spectra (A) are also shown for the free (O——O) and bound dye (· · · ·). This function is independent of wavelength for a species with constant quantum yield and polarization of fluorescence in the absence of energy transfer. *All data for the free dye are shown on a 10-fold expanded scale.* Spectra were obtained in 1.0 M NaCl (pH 7.0) containing either 51 μM ethidium bromide and/or 107 μM $(dG\text{-}dC)_{\overline{200}}$ in cuvettes with a 5-mm light path. The fraction of bound dye was calculated to be 0.39 from the difference spectra at 284 and 470 nm. Fluorescence excitation and emission spectra were instrumentally corrected to constant illumination intensity and overall detector sensitivity. They were additionally corrected for self-absorption effects but not for variation in emission polarization as a function of excitation wavelength. Spectra for the bound dye were derived from measured curves by correction for the presence of unbound dye. The emission was measured at 600 nm for excitation spectra; emission spectra were obtained with excitation at 511 nm. (*Ordinate* is given in arbitrary units.)

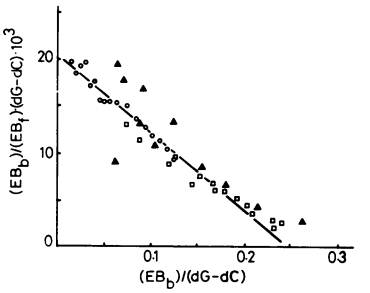


FIG. 2. Scatchard plot for binding of ethidium bromide to $(dG\text{-}dC)_{\overline{200}}$ as determined by different methods in 1.0 M NaCl. O, Titration of $(dG\text{-}dC)_{\overline{200}}$ with ethidium bromide (EB) and measurement of the relative decrease of absorption at 284 nm. □, Titration of EB with increasing amounts of $(dG\text{-}dC)_{\overline{200}}$ and calculation of the amount of free (EB_f) and bound (EB_b) from the change of absorption at 470 nm. ▲, Binding studies in an analytical ultracentrifuge at 20° and 40,000 rpm. All concentrations are molar in dye or nucleotide. Polymer concentrations in absorbance measurements were between 20 and 600 μM.

increases greatly–about 15-fold at 600 nm—and the emission peak is blueshifted about 10 nm, a finding not reported for natural DNA (4). The corrected excitation spectra correlate well with the absorption spectrum for the free dye (see ratio in Fig. 1*b*), but there is clear evidence for energy transfer from DNA in the ultraviolet, as best seen by the peak at 255 nm for the ratio. The excitation peak at 520 nm for the bound dye is, however, consistent with the derived absorption spectrum. Part of the variations in the apparent quantum yield are due to polarization changes (Fig. 1*b*). The optical properties of bound ethidium bromide at high-salt concentrations are very similar to those at 1 M NaCl.

Ethidium bromide cosediments with poly(dG-dC) upon centrifugation. This observation constitutes more direct physical evidence for the binding process. The sedimentation coefficient of $(dG\text{-}dC)_{\overline{200}}$ decreases by about 20% upon saturation with ethidium bromide.

Equilibrium data obtained by different methods for binding of ethidium bromide to $(dG\text{-}dC)_{\overline{200}}$ in 1 M NaCl are given in Fig. 2 as a Scatchard plot. The binding isotherm is a straight line, a finding consistent with a simple binding phenomenon, as is the occurrence of a single relaxation process in temperature-jump experiments. The data in Fig. 2 were obtained at low ethidium bromide concentrations in order to avoid corrections for self-association of the dye in solution. (If a dimerization model is assumed, the corresponding equilibrium constants were determined to be about 2.1 mM in 1 M NaCl and 0.8 mM in 4.4 M NaCl.) The dissociation constant of ethidium bromide from poly(dG-dC), as determined from the slope of the Scatchard plot, is 12 ± 2 μM referred to the concentration of binding sites. This value is consistent with an independent determination in preliminary relaxation experiments and also agrees with data reported for natural DNA of different base composition (4).

The stoichiometry of binding obtained by optical titration, gel filtration, and analytical centrifugation is one molecule of ethidium bromide per 2.1 ± 0.2 base pairs.

Repetition of these measurements in 4.4 M NaCl, where poly(dG-dC) is in the *L*-form, leads to strikingly different binding behavior of ethidium bromide. Fig. 3*a* gives as an example the differences in the absorption of solutions containing different amounts of $(dG\text{-}dC)_{\overline{200}}$ and at various concentra-

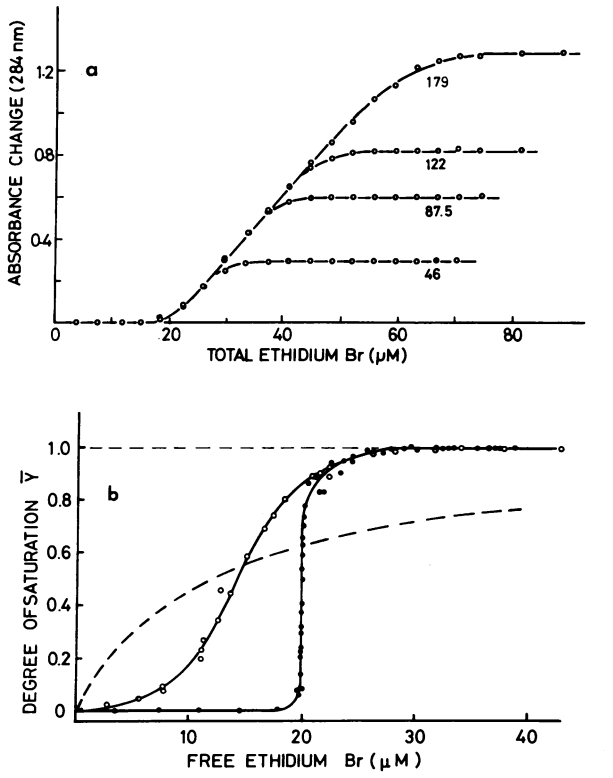


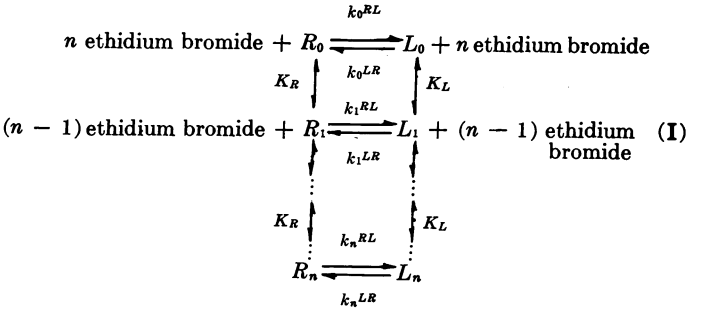
FIG. 3. Cooperative binding of ethidium bromide poly(dG-dC) at high-salt concentration. (*a*) Difference of absorbance at 284 nm of ethidium bromide between a solution containing the indicated $(dG\text{-}dC)_{\overline{200}}$ concentrations in μM and one without the polynucleotide as a function of total ethidium bromide concentration. The solutions contained 4.4 M NaCl (pH 5.6–7.0). Under these conditions, poly(dG-dC) is in the *L*-form. No binding is observed until the ethidium bromide concentration reaches about 20 μM. The total change in the extinction coefficient upon binding of ethidium bromide is $\Delta\epsilon_{284} = -30{,}000\ M^{-1}\,cm^{-1}$. This value is compatible with the sum of the individual contributions from ethidium bromide and poly(dG-dC) under the assumption that the latter undergoes the transition from *L*- to *R*-form (2). (*b*) Degree of saturation or fraction of occupied sites, $\bar{Y}$, as a function of *free* ethidium bromide concentration at two salt concentrations. Titrations at the high-salt concentration were performed by stepwise addition of the dye and waiting until the change of fluorescence or absorption reached equilibrium. (– – –): $(dG\text{-}dC)_{\overline{200}}$, 1.0 M NaCl; (●——●): $(dG\text{-}dC)_{\overline{200}}$, 4.4 M NaCl; (○——○): $(dG\text{-}dC)_{\overline{10}}$, 4.4 M NaCl.

tions of ethidium bromide. In Fig. 3*b*, the degree of saturation that is, the number of bound molecules divided by the number of binding sites, is plotted against the free ethidium bromide concentration as measured by absorption and fluorescence. At high-salt concentration, markedly sigmoidal binding curves are observed that are independent of the $(dG\text{-}dC)_{\overline{10}}$ and $(dG\text{-}dC)_{\overline{200}}$ concentrations over a 100-fold range. These data also show a pronounced dependence on chainlength; for $(dG\text{-}dC)_{\overline{200}}$ a nearly stepwise binding curve is obtained. The number of binding sites, however, is practically the same as at low-salt concentration.

When the equilibrium data are plotted as a "Hill plot", that is, log $[\bar{Y}/(1-\bar{Y})]$ against log (ethidium bromide$_{free}$), they yield, at 1 M NaCl, a straight line with the expected slope equal to one. At high-salt concentration, the maximal slope is 6 for $(dG\text{-}dC)_{\overline{10}}$. The maximal slope could not be determined for $(dG\text{-}dC)_{\overline{200}}$, but it is larger than 40, reflecting the extremely high cooperativity and molecular weight dependence of this binding process. *Thus, with* $(dG\text{-}dC)_{\overline{200}}$ *a greater than 40th power dependence on the concentration of free dye is observed.*

Allosteric Model. Different models can account for the cooperative ligand binding described above and have been extensively discussed for proteins (10–12). Simple models involving a strong initial binding to the *L*-form without a change of the optical properties of the bound molecule are excluded by the lack of dependence upon the polymer concentration. The simplest model that gives an apparent agreement with the experimental data is the "allosteric model" (13–15). Poly-(dG-dC) assumes two conformations, *R* and *L*, and the transition between them is cooperative and concerted (2).

If the binding constants are the same for all sites on a molecule in a particular conformation, but different for the two forms, *R* and *L*, the reaction scheme is given by



The vertical columns are the binding equilibria, with the dissociation constants of EB corresponding to the *R*-form and the *L*-form equal to K_R and K_L, respectively. The number of binding sites per molecule is *n*, and the rate constants in the horizontal direction are the overall rate constants for the conformational change between the two forms. The assumption of equal binding sites agrees with experimental results at low-salt

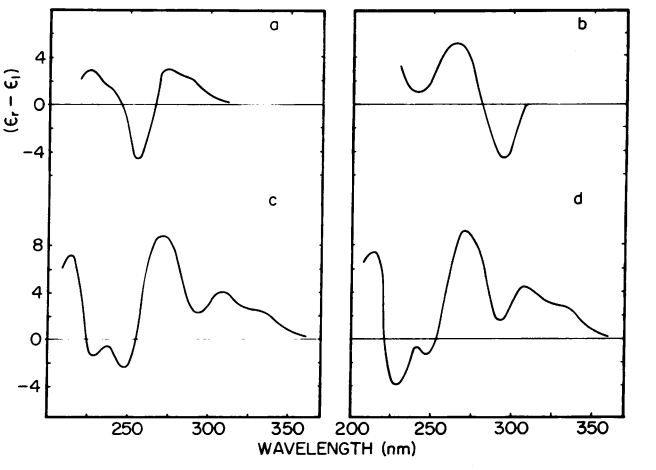


FIG. 4. Circular dichroism spectra of $(dC\text{-}dG)_{\overline{10}}$ in solutions with different salt and ethidium bromide concentrations. (*a*) 1.0 M NaCl, no dye (R_0-form); (*b*) 4.4 M NaCl, no dye (L_0-form); (*c*) 1.0 M NaCl, 40–50 μM ethidium bromide; (*d*) 4.4 M NaCl, 40–50 μM ethidium bromide. The ethidium bromide concentrations used in (*c*) and (*d*) will saturate most of the binding sites on the polynucleotide. [Values of $(\epsilon_r - \epsilon_l)$ are based on nucleotide concentration.]

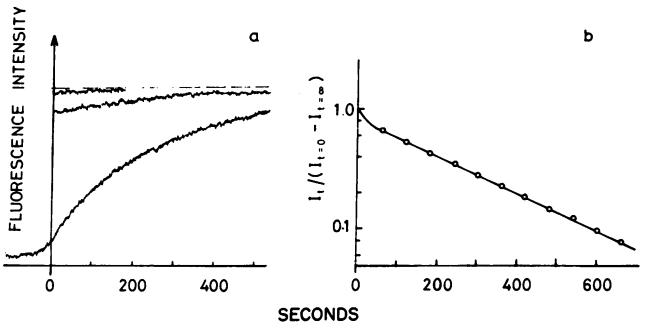


FIG. 5. (*a*) Time-dependent change of the fluorescence intensity, I_t, after stopping the continuous linear addition of a total of 10 μl of a 1.66 mM ethidium bromide solution for 2 min to 3.0 ml of 2.5 μM (dG-dC)$_{\overline{10}}$ giving a final concentration of 17.1 μM ethidium bromide in 4.4 M NaCl at 30°. (*b*) Semilogarithmic plot of the data (*a*) according to a first-order reaction. The reciprocal slope corresponds to 2.303τ, where τ is the relaxation time that, according to mechanism **I**, describes the isomerization of the nucleic acid.

concentration. For molecules with a small number of binding sites, however, it may become necessary to modify this assumption to take into account end-effects.

From this model, the formalism already developed for oligomeric proteins is easily applied (13–17). For example, the degree of saturation, $\bar{Y}$, is given by (13):

$$\bar{Y} = \frac{K^\circ_{RL} \cdot c\alpha(1 + c\alpha)^{n-1} + \alpha(1 + \alpha)^{n-1}}{K^\circ_{RL}\,(1 + c\alpha)^n + (1 + \alpha)^n} \qquad \textbf{(II)}$$

where $c = K_R/K_L$ is the nonexclusive binding coefficient, α = ethidium bromide$_f/K_R$, the normalized free ethidium bromide concentration, and K°_{RL} is the allosteric constant, i.e., the ratio of L_0/R_0 in the absence of ethidium bromide. The ratio L_0/R_0 itself depends upon salt concentration and the chain length of the polymers (2).

Qualitatively, the binding data at high-salt concentration can be interpreted as follows: ethidium bromide binds only weakly to the *L*-form but strongly to the *R*-form, and in this way shifts the conformational equilibrium to the *R*-form. Two of the predictions following from such a mechanism are: (*i*) At saturating ethidium bromide concentration, i.e., $\alpha \gg 1$ and $\alpha^n \gg K^\circ_{RL}$, we expect from the mass action law that most of the molecules will be in the R_n-form if $c \ll 1$. Therefore, the structure of the complex of poly(dG-dC) with ethidium bromide under these conditions should be the same at high- or low-salt concentrations. (*ii*) The first-order kinetics of the conformational change should be observable by the change of absorption or fluorescence, since appreciable binding will occur only after molecules have changed to the high affinity *R*-form. Based upon previous kinetic results for the $R_0 \rightleftharpoons L_0$ transition, we expect changes in the optical properties of ethidium bromide to occur in the range of minutes.

Circular Dichroism Data. To check prediction (*i*) of this model, we measured CD spectra, since they are sensitive indicators of helical structures in solution. Measurements were made in the absence and at near saturating concentrations of ethidium bromide (Fig. 4). In 1.0 and 4.4 M NaCl, the spectra are different in the absence of ethidium bromide, but they become similar at high concentrations of the dye. Small differences between spectra (*c*) and (*d*) of the complex in the presence of low- and high-salt concentrations, respectively, may be due to the different solvent conditions. This substantiates the expectation that the complex has similar structures whether one works under conditions where the *R*- or the *L*-form prevails in the absence of the drug. Such a conclusion can also be drawn from the overall change in absorbance upon binding at high-salt concentration (Fig. 3*a*).

Kinetic Experiments. An example of the time-dependent change of fluorescence at high-salt concentration after addition of ethidium bromide is shown in Fig. 5*a*. The reaction is represented mostly by a simple exponential curve. From $dI_t/dt = (I_{t=0} - I_{t=\infty}) \exp(-t/\tau)$, the relaxation time, τ, is obtained from the slope of a semilogarithmic plot (Fig. 5*b*). The relaxation times are 10^2–10^3 sec and are independent of the poly(dG-dC) concentration over a 20-fold range. This result is expected if the rate-determining step is the conformational change of the nucleic acid, occurring as an "all-or-none" transition (2).

If all binding processes are fast compared to the conformational changes of the polynucleotide, as indicated by temperature-jump experiments at 1 M NaCl of the binding to the *R*-form, the relaxation time, τ, for the isomerization at high ratios of ethidium bromide to nucleic acid (buffered in ethidium bromide) is related in a simple way to the ethidium bromide concentration (17):

$$\tau^{-1} = k_0^{RL} \frac{(1 + \alpha\delta)^n}{(1 + \alpha)^n} + k_0^{LR} \frac{(1 + \alpha\delta)^n}{(1 + c\alpha)^n} = k^{RL} + k^{LR} \qquad \textbf{(III)}$$

where $\delta = k_{i+1}^{RL}/k_i^{RL} = c \cdot k_{i+1}^{LR}/k_i^{LR}$, the ratio of the rate constants for the conformational change upon binding of an additional dye molecule. If end-effects must be taken into account, the dependence of the kinetics of the conformational change on the concentration of ethidium bromide will be more complicated. It is expected that such end-effects will mainly affect the nucleation reaction of the transition (2).

The results of kinetic experiments at different ethidium bromide concentrations for (dG-dC)$_{\overline{10}}$ in 4.4 M NaCl are

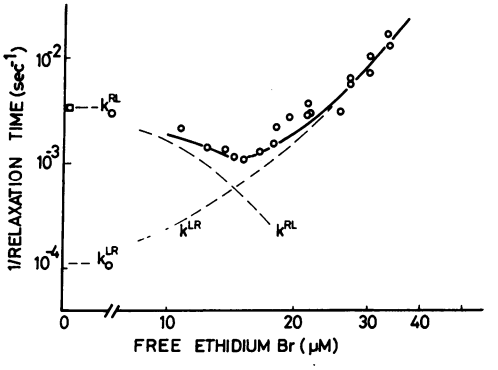


FIG. 6. Relaxation time, τ, for the change of fluorescence of (dG-dC)$_{\overline{10}}$ as a function of the final concentration of free ethidium bromide in 4.4 M NaCl. The functional dependence of the overall rate constants k^{LR} and k^{RL}, which describe the time dependence of the isomerization, are indicated by *dashed lines*. High ratios of ethidium bromide to nucleotide were used, and the equilibrium was shifted by addition of small amounts of dye. The value of k_0^{RL} (□) in the absence of ethidium bromide was measured directly by the increase of absorption of (dG-dC)$_{\overline{10}}$ at 295 nm after the salt concentration was increased to 4.4 M NaCl (2).

collected in Fig. 6. The reciprocal relaxation time shows a minimum near half-saturation; it increases with the 4th power at high-dye concentration and extrapolates at low ethidium bromide concentration to a value that is the same as that obtained from direct measurements of the transition kinetics by shifts in the salt concentration (2). Such a correspondence of the extrapolated relaxation time with the one measured directly in the absence of ethidium bromide strongly supports the proposed mechanism.

Due to the heterogeneity of $(dG\text{-}dC)_{\overline{10}}$, no detailed fit of the experimental data to the model was attempted. Instead, model calculations were performed on a computer, where all the parameters of Eqs. **I** and **III** were systematically varied in turn. These were visually compared with the binding and kinetic results given in Figs. 3*b* and 6. Reasonable agreement for τ and $\bar{Y}$ as functions of the concentration of free ethidium bromide was obtained with $K^{\circ}_{RL} = 50\text{–}100$, $K_R = 20$ μM, $c < 0.01$, and $\delta = 0.3\text{–}0.5$.

The "allosteric constant," $K^{\circ}_{RL} = k_0^{RL}/k_0^{LR}$, agrees with the one determined directly from the salt-induced transition (2). Since K_R shows no salt dependence between 0.6 and 2.0 M NaCl, a value of 20 μM for 4.4 M NaCl is reasonable. The parameter, $K_L = K_R/c > 2$ mM, implies that binding of ethidium bromide to the *L*-form is weak or nonexistent and is consistent with the observation that at 40 μM ethidium bromide no initial fast binding could be detected after the *L*-form was mixed with ethidium bromide.

At high degrees of saturation ($\bar{Y} > 0.5$) a systematic deviation of the model calculation from the experimental data (Fig. 3*b*) could not be removed. A complete, quantitative description of the data, therefore, may require an extension of the proposed "concerted" model to include a "linear" mechanism, such as proposed by Koshland *et al.* (11), in which end-effects can be treated.

DISCUSSION

We show here that the effect of simple ions on the conformation of a synthetic DNA, poly(dG-dC), can be reversed by interaction with ethidium bromide. The proposed allosteric model for this interaction describes experimental results such as the sigmoidal binding curves, the CD spectra, and the kinetics of binding and conformational transition. More complicated models may become necessary if end-effects are taken into consideration, but the one proposed serves as a convenient and simple starting point for the description of cooperative binding. The extremely high degree of cooperativity is the result of the exclusive nature of the binding process ($c \ll 1$) and the large number of binding sites on a given polynucleotide.

Preliminary experiments with proflavine and quinacrine·HCl do not show effects comparable to the cooperative binding of ethidium bromide to poly(dG-dC) at high-salt concentration. With regard to the staining pattern of chromosomes, one can imagine parts of the DNA being in a low affinity form for certain dyes due to structural differences. Such parts may switch to a high affinity form only at drug concentrations above certain thresholds. These speculations have to be checked with DNA under more physiological conditions, such as in chromosomes, since factors other than salt, e.g., nucleoproteins (18), may be critical in establishing the structure of polynucleotides.

The observation that DNA with a certain base sequence can react in a specific and cooperative way with small molecules, like ions or ethidium bromide, may serve as one possible model for the regulation of gene expression on the basis of the DNA structure itself. It is conceivable that parts of the DNA in a cell change their conformation from an active to an inactive state or *vice versa* by cooperative interactions with small molecules or proteins.

This work was supported by an EMBO long-term fellowship to F. M. P. during his stay at the Molecular Enzymology Laboratory, University Bristol and a grant by the Royal Society, London to J. J. H. for building the differential fluorimeter with automatic titration attachment. This work was done during the term of an Established Investigatorship of T. M. J. from the American Heart Association.

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