Hydrogen–Deuterium Exchange Analysis of Ligand–Macromolecule Interactions: Ethidium–Deoxyribonucleic Acid System†

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ABSTRACT: The interaction between DNA and the intercalating dye, ethidium bromide, was studied by use of a novel approach in which hydrogen–deuterium (H–D) exchange between ethidium amino groups and solvent was measured spectrophotometrically in a stopped-flow mode. The method depends on the fact that ethidium H exchange is greatly slowed on complexation with DNA and that H–D exchange kinetics of the chromophore can be monitored via an accompanying change in its spectral absorbance. The H–D exchange dependent spectral character of ethidium was characterized, and the catalyzed exchange behavior of the free dye and the dye–DNA complex was studied. From such measurements, one can obtain rate and equilibrium constants for the interaction and possibly also some stereochemical information. The constants obtained were checked in more conventional mixing experiments. At 20 °C in high salt, the equilibrium binding constant is $\sim 5 \times 10^4$ M^{-1}, and on and off rate constants are 1.6×10^6 M^{-1} s^{-1} and 30 s^{-1}, respectively. The results independently confirm that one dye molecule is bound for each 2–2.5 base pairs. The method should be applicable to a range of binding interactions. Among other advantages, this approach can allow tight binding interactions to be studied at concentrations of the reactants far above the characteristic K_{diss} value.

This paper reports on studies of the intercalative binding of the trypanocidal dye ethidium bromide to DNA. In stopped-flow experiments, we monitored the hydrogen–deuterium exchange of amino protons in ethidium (structure shown in Figure 2) by means of an absorbance change in the visible spectrum near 540 nm. The results yield equilibrium and kinetic constants for complexation of ethidium by DNA, and these constants were validated in more conventional stopped-flow mixing experiments.

Information about complexes between ligands and macromolecules has been most often derived from binding studies that rely either on partitioning free ligand from bound ligand or on establishing a spectroscopic feature of the complex that is distinct from the free forms. The present work shows that the stopped-flow kinetic measurement of exchangeable hydrogens of a chromophoric ligand can be used to determine both equilibrium and kinetic parameters of complexation and may indirectly provide information about the stereochemistry of binding interactions in the complex. An analogous approach, using radioisotope techniques to follow tritium exchange of the S-peptide on binding to ribonuclease, has been reported by Schreier & Baldwin (1976). A similar application to the subunit dissociation of hemoglobin has also been carried out (Barksdale & Rosenberg, 1978). The spectral method described here should be generally applicable to a number of interactions important for both proteins and nucleic acids.

Detection of H exchange in a chromophoric molecule by differential ultraviolet or visible absorbance takes advantage of the fact that exchange of a vicinal proton for a deuteron is quite generally accompanied by a change in electronic energy levels. The effect is due to the fact that the zero-point vibrational energy contributes to the difference in energy between the electronic ground and excited states of the chromophore. Previous studies have documented spectral changes in the ultraviolet accompanying H–D exchange of both ring and amino protons of nucleic acid bases (Cross, 1975; Nakanishi & Tsuboi, 1978a,b; Mandal et al., 1979) as well as the amide proton in peptide bonds (Takahashi et al., 1978; Englander et al., 1979). A corresponding change in the visible-region absorption spectrum of ethidium bromide arising from H–D exchange is reported here.

Materials and Methods

(*1*) *Materials.* Calf thymus DNA and deuterium oxide (99.8%) were purchased from Sigma Chemical Co. The highly polymerized DNA sample was sonicated to reduce its viscosity. This was done by repeatedly applying short bursts of sonication with a Branson sonifier to the sample in ice with several minutes of cooling in between. The concentration of DNA in solution was measured by use of the molar extinction coefficient, 6.7×10^3 M^{-1} cm^{-1}, at 260 nm (Hanson, 1971). Highly purified ethidium bromide was obtained from Boehringer Mannheim GmbH. The dye concentration was measured spectrally by using 5.65×10^3 M^{-1} cm^{-1} as the molar extinction coefficient at 480 nm (Waring, 1965). This value differs by less than 4% from that used by Bresloff & Crothers (1975) which corrects for residual ethanol. All other reagents were analytical reagent grade.

(*2*) *Stopped-Flow Spectrophotometry.* Stopped-flow kinetic experiments were performed with a stopped-flow spectrophotometer system designed by OLIS Inc. of Jefferson, GA. The optical cell and photomultiplier together with drive syringes and provision for temperature control were from Durrum. The light source, a 150-W xenon arc, was used with a Bausch & Lomb 2-m monochromator at 1-mm slit width. Interfacing of a NOVA 2 computer to the stopped-flow instrument and all the software was done by OLIS. A computer program for analyzing kinetic traces was modified from one obtained from Dr. Brian Sykes. The dead time of the instrument was found to be 3 ms from the reaction of ferric nitrate and potassium thiocyanate in 0.1 N H_2SO_4 solution. Drive syringes with ratios 1:1 and 1:9 were used. The drive syringes, flow system, and observation chamber were thermostated and maintained within ±0.2 °C of the desired temperature by a circulating pump.

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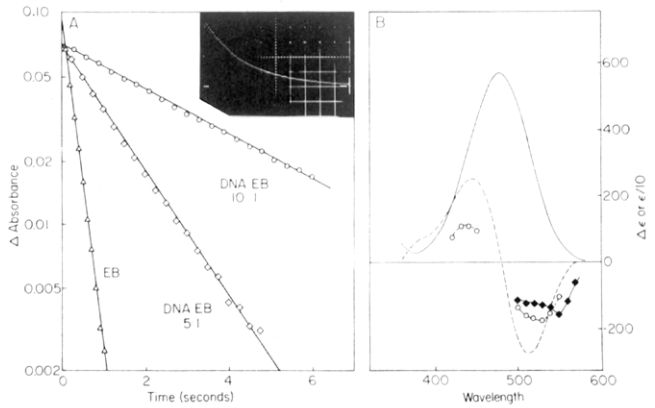

FIGURE 1: (A) Semilogarithmic plot of the kinetic traces followed at 540 nm resulting from mixing ethidium or its DNA complex in H_2O buffer with D_2O buffer of the same composition. The buffer solution contained 1 M NaCl, 1 mM phosphate, and 0.6 mM ethidium; the temperature was 20 °C. The insert shows a photograph of a typical oscillograph trace arising from H–D exchange of ethidium amino protons. (B) Equilibrium and kinetic difference spectra of free and bound ethidium bromide: (—) absorbance spectrum (reduced 10-fold in scale) of free ethidium; (- - -) the equilibrium difference spectrum of 0.1 mM ethidium between D_2O and H_2O buffer solutions; (○) the transient kinetic difference spectrum due to exchange of ethidium amino protons on transfer of 0.6 mM dye from H_2O to D_2O solution; (♦) kinetic difference spectrum of the ethidium–DNA complex, with 0.6 mM ethidium and 3 mM DNA–P. All spectra were taken in 0.1 M NaCl and 1 mM phosphate, pH 9, at room temperature.

(*3*) *Difference Spectral Measurements.* Equilibrium difference spectra were recorded on a Cary 118 spectrophotometer in the wavelength range between 360 and 580 nm by carefully diluting a concentrated solution of the sample into sets of H_2O and D_2O buffers of identical composition. Dilution error was less than 1%, and average values of four different dilution pairs were taken to minimize the statistical error. Kinetic difference spectra were obtained by measuring the amplitudes of kinetic traces at different wavelengths with the stopped-flow instrument.

Results

(*1*) *Kinetic and Spectral Characteristics of the H–D Exchange of Ethidium Bromide.* (*a*) *Kinetic Trace and Assignment of Protons.* A stopped-flow kinetic trace obtained by mixing ethidium bromide in H_2O buffer with an equal volume of D_2O buffer is shown in Figure 1A. The CRT photo shows computer-recorded and averaged data points from five sequential stopped-flow shots. The time-dependent change in absorbance reflects reequilibration of the four hydrogens in the amino groups of the dye molecule with solvent deuterium. A semilog plot of the data is monophasic over four to five half-times. Though the amino groups in ethidium bromide are not chemically equivalent (Figure 2), the exchange of all four protons was kinetically identical under all conditions of pH and catalyst concentration tested.

(*b*) *Equilibrium and Kinetic Difference Spectra of Free and Complexed Dye.* Figure 1B compares the absorption spectrum of ethidium bromide with the difference spectra that result when it is transferred from H_2O into D_2O. The absorption spectrum is plotted on a 10-fold-compressed scale. The overall equilibrium difference spectrum is fairly symmetrical (peak at 445 nm; trough at 510 nm), indicating a simple blue shift in D_2O by ~5 nm. This spectrum is made up of a kinetically distinguishable contribution due to H–D exchange and a solvent perturbation contribution of approximately equal size. The kinetic difference spectrum displays a peak at 435 nm and a trough at 530 nm. We used the large H–D exchange dependent change in absorbance at 540 nm (~12%) to obtain kinetic traces with a good signal to noise ratio for ethidium bromide concentrations of 0.1–1 mM. The kinetically determined spectral change accompanying H–D exchange of ethidium when it is complexed with calf thymus DNA is also shown in Figure 1B.

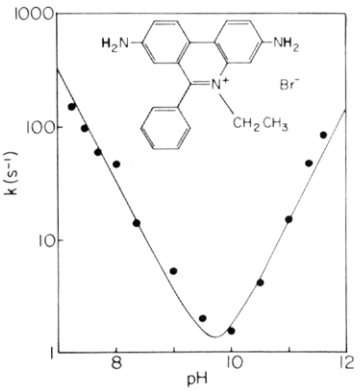

FIGURE 2: pH dependence of the H–D exchange rate of free ethidium bromide. Rates were determined from absorbance differences at 540 nm measured in solutions containing 0.1 M NaCl, 1 mM phosphate, and 0.5 mM ethidium bromide, at 20 °C, titrated to the apparent pH value indicated. Below pH 10, the exchange of the amino groups is acid catalyzed.

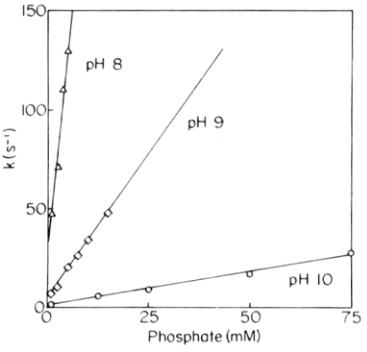

FIGURE 3: Catalysis of H–D exchange of free ethidium by (acid) phosphate. These experiments were performed by adding the indicated concentrations of phosphate to a solution of 0.1 M NaCl, 1 mM phosphate, and 0.5 mM ethidium, at 20 °C, in D_2O and H_2O in the stopped-flow instrument. Traces were measured at 540 nm.

The visible spectrum of the dye is shifted to the red on complexation. The kinetic difference spectrum (D minus H) of complexed dye also is shifted to the red when compared to that of the free dye.

(*2*) *H–D Exchange of Free Ethidium Bromide.* (*a*) *Effect of pH.* The effect of pH on the H–D exchange of ethidium bromide was determined between pH 7 and pH 12. The sample in D_2O was mixed with H_2O buffer in the volume ratio 1:9. A plot of log rate constant against pH (Figure 2) demonstrates catalysis of the exchange by H^+ ion, with a catalytic constant of 3.0×10^9 M^{-1} s^{-1} (near the diffusion-controlled limit) and by OH^- ion with a constant of 1.8×10^4 M^{-1} s^{-1}. Evidently the rate-limiting H^+ ion catalyzed step involves direct protonation of an amino group (pK ~2.5). Catalysis by OH^- presumably involves direct deprotonation to form the ethidium anion.

(*b*) *Effect of Catalysts.* Phosphate was found to catalyze the H–D exchange of ethidium bromide (Figure 3). The increase in catalytic effect with decreasing pH indicates that the acid phosphate ion ($H_2PO_4^-$) is the effective catalytic form, according to (see Table I)

$$k_{ex} = k_0 + k_{cat}[HP] \quad (1)$$

Table I: Dependence of H–D Exchange Rate Constants of Ethidium Bromide on the Acid Form of Phosphate Catalyst[a]

fraction of $H_2PO_4^-$	pH	rate constant (total P) (M^{-1} s^{-1})	rate constant ($H_2PO_4^-$) (M^{-1} s^{-1})
0.074	8.0	2.2×10^4	3.0×10^5
0.0079	9.0	3.0×10^3	3.8×10^5
0.00079	10.0	3.4×10^2	4.3×10^5

[a] Conditions: 0.1 M NaCl; 0.5 mM ethidium bromide; 20 °C; 540 nm (data in Figure 4).

Table II: Binding Constant from H–D Exchange of DNA–EBr Complexes Measured with Different Ratios of Binding Sites to Ethidium Bromide

DNA (mM)	EBr (mM)	rate constant (s^{-1})	equilibrium constant (M^{-1}) × 10^{-4} $k_b = 0.0$	equilibrium constant (M^{-1}) × 10^{-4} $k_b = 0.2$ s^{-1}
0.0	0.60	3.69		
0.75	0.60	2.84		
1.50	0.60	2.03	2.7	5.9
2.25	0.60	1.26	3.5	6.8
3.00	0.60	0.71	3.6	6.4
6.00	0.60	0.25	2.4	
7.25	0.30	0.21	1.5	
9.15	0.25	0.19	1.2	

The data define a catalytic constant (k_{cat}) for acid phosphate of 3.7×10^5 M^{-1} s^{-1}. Since the pK values of phosphate and ethidium differ by ~4.5 units, this is close to the ideal diffusion-limited rate constant (Englander et al., 1972). Similar experiments with cacodylic acid at pH 9 suggest that it is intrinsically 10 times less effective as a catalyst than phosphate.

(3) DNA–Ethidium Complex. (a) H–D Exchange and Catalysis. The kinetics of H–D exchange must be responsive to DNA binding in order to serve as a probe of complexation. This behavior is illustrated in Figure 1A which compares the H–D exchange behavior of free ethidium with results for complexes at differing ratios of DNA to ethidium bromide. Binding of the drug to DNA leads to retardation of H–D exchange rate, with the linear plots indicating first-order monophasic decays. Manipulation of this system allows determination of the DNA–ethidium equilibrium binding constant as well as the association and dissociation rate constants. The dependence of the measured H–D exchange rate on concentration of DNA is shown in Figure 4. These data permit estimation of the binding constant (see Discussion and Table II).

Catalysis of H–D exchange rate in the complex by phosphate at pH 6.5 and pH 7.5 is shown in Figure 5. It can be seen that just as the H–D exchange rate of free dye is accelerated by increasing phosphate, H–D exchange in the dye–DNA complex initially increases, but it asymptotes to a constant value at higher phosphate concentration. This limiting rate is only slightly sensitive to pH, being 27 s^{-1} at pH 7.5 and 32 s^{-1} at pH 6.5. The limiting rate represents the dissociation rate of the dye–DNA complex (see Discussion).

It can also be concluded that phosphate catalysis of the H–D exchange rate of bound ethidium is much less effective (~1/50) than for free dye molecules.

(b) Direct Observation of Binding Kinetics. In the favorable case of the ethidium–DNA complex, the red shift of the drug molecule accompanying binding is great enough to permit the kinetics of association to be followed directly by stopped-flow spectrophotometry. To do this, we performed a series of concentration jump experiments in which DNA and ethidium

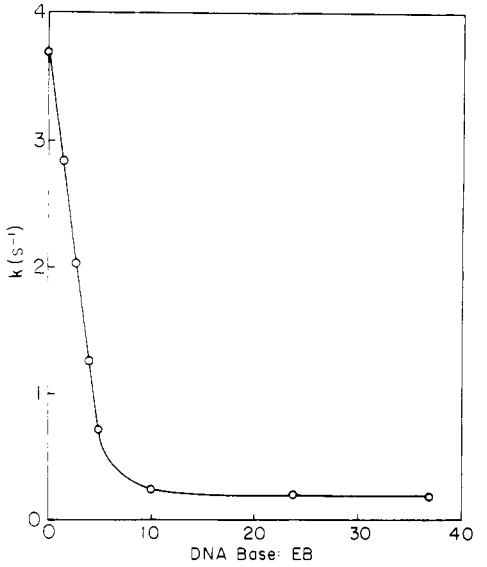

FIGURE 4: Influence of the extent of binding of ethidium to DNA on the H–D exchange rate of ethidium bromide. These experiments were performed at 20 °C, pH 9, in solutions containing 1 M NaCl and 1 mM phosphate, with from 0.25 to 0.6 mM ethidium and 0 to 9.15 mM DNA–P, as indicated in Table II.

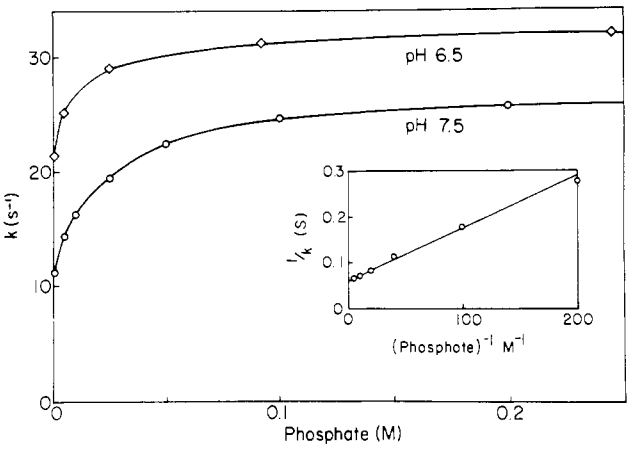

FIGURE 5: Catalysis by phosphate of the H–D exchange rate of ethidium complexed to DNA. The final ethidium concentration was 0.6 mM, and calf thymus DNA was present at 3 mM P in solutions containing 1 M NaCl and 1 mM phosphate at the indicated pH values and 20 °C. The inset shows a double-inverse plot of the excess exchange rate at pH 7.5 as a function of the phosphate concentration, according to eq 7b.

in H_2O buffer were diluted 10-fold by mixing with an excess of H_2O buffer in the stopped-flow instrument. In these experiments, the ratio of ethidium to DNA phosphate was fixed at 1:5 while the initial concentration of the complex was varied. The time course of relaxation was followed by change in absorbance at 480 nm. A typical trace is shown in Figure 6A along with its semilogarithmic plot. The relaxation was first order and monophasic over the range of concentrations of DNA and ethidium bromide we investigated. Since we were able to follow the relaxation over a time range in excess of 3τ, the use of the large ΔC perturbation in the linear relaxation approximation below can be justified (Bernasconi, 1976, Chapter 5).

If we assume the simplest bimolecular mechanism for the reaction of ethidium and DNA sites, i.e.

$$\text{EB–DNA} \underset{k_{+1}}{\overset{k_{-1}}{\rightleftharpoons}} \text{EB} + \text{DNA} \qquad (2)$$

the inverse of the relaxation time (τ) measured in this ex-

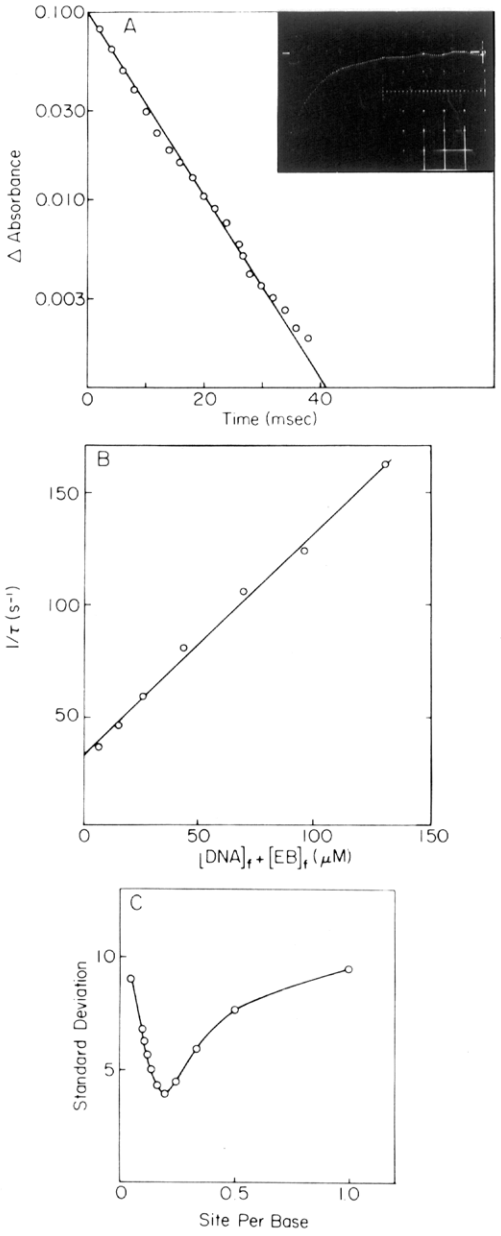

FIGURE 6: Direct stopped-flow spectrophotometric analysis of the binding of ethidium to DNA. (A) Insert: a kinetic trace at 480 nm obtained on 10-fold dilution of an ethidium–DNA complex (final concentrations = 0.072 mM ethidium and 0.36 mM DNA–P). The solution contained 1 M NaCl and 1 mM phosphate at pH 6.5 and 20 °C. A semilogarithmic plot of the data from the dilution trace gives the line shown in the body of panel A. (B) The reciprocal of the relaxation time measured at 480 nm as a function of equilibrium concentrations of free ethidium and DNA sites (DNA_f). Conditions were identical with those of panel A. Linearity of this graph is predicted by eq 3 of the text if the correct site size is used. The graph shown assumed a site size of 5 P/site. (C) Minimal standard deviations resulting from assuming eq 3 and different values for the site size in DNA occupied by ethidium. The calculation is described in the text and provides a method for determining binding site size.

periment can be expressed as in eq 3 [see Bernasconi (1976) for example]:

$$1/\tau = k_{+1}([DNA]_f + [EB]_f) + k_{-1} \quad (3)$$

Here $[DNA]_f$ and $[EB]_f$ are the concentrations of free DNA sites and free ethidium at equilibrium. In order to fit the relaxation times in the equation, one requires the free concentrations at equilibrium, which assumes knowledge of the equilibrium constant. We proceeded by the following iterative method (Bernasconi, 1976). Initial approximate values of k_{+1} and k_{-1} were estimated by using the initial concentrations of the reactants; this gives an approximate value of the equilibrium constant. This value in turn can be used to estimate the free concentrations. Repeated iteration yields successive sets of the constants finally reaching a set of constant values approaching the actual values. In a seven-point experimental set, the refinement showed no further change in the fourth decimal of the rate constants after 16 iterations, and this required only a few seconds of time on a NOVA-2 computer. The resulting values of the iteration give the following values for the rate and equilibrium constants:

$$k_{+1} = 1.01 \times 10^6\ M^{-1}\ s^{-1}$$

$$k_{-1} = 31.5\ s^{-1}$$

$$K = 3.21 \times 10^4\ M^{-1}$$

In performing this calculation, one must assume a DNA binding site size. The calculation then leads to values for k_{+1}, k_{-1} and K best matching the whole data set. Given these values, one can plot measured $1/\tau$ against $([DNA]_f + [EB]_f)$ (Figure 6B). Equation 3 requires that this plot be a straight line. When the initially assumed site size is incorrect, a curved line results. The best fit to a straight line (Figure 6B) was obtained with a site size equal to five bases. This test is shown in Figure 6C which plots, against assumed site size, the standard deviation of the plot when the best straight line is drawn through the points. The same binding site size of 2.5 base pairs was determined in equilibrium binding experiments by LePecq & Paoletti (1967) and has been put forward for other intercalative drugs as well [see Neidle (1979)].

An adjustment of the apparent value of k_{+1} obtained in these experiments can be applied to correct for the effect of removal of lattice sites as a consequence of the site exclusion (McGhee & von Hippel, 1974). For example, one or two phosphates occurring between two noncontiguous ethidium molecules are no longer available. The intrinsic value of k_{+1} is then underestimated by a factor $[(1 - n\nu)/[1 - (n-1)\nu]]^{n-1}$ (McGhee & von Hippel, 1974), where n is the site size and ν is the fraction of DNA occupied. When this factor is applied to the data, which correspond to coverage of 4–27% of the lattice, k_{+1} increases by 60% and is $1.6 \times 10^6\ M^{-1}\ s^{-1}$, with the same factor applying to K, which then represents the intrinsic site affinity, not the apparent value. The corrected value of K then is 5.1×10^4.

The association rate constant (k_{+1}) was also measured independently by direct mixing of a solution of ethidium with an equal volume of DNA solution containing a 5.6-fold excess of DNA "sites". Under these conditions, the reaction is pseudo first order. The final total concentrations of DNA and ethidium were 0.95 mM (P) and 0.034 mM, respectively, in H_2O buffer containing 1.0 M NaCl and 1 mM phosphate, pH 6.5, and 20 °C. The pseudo-first-order rate constant for loss of free ethidium was found to be $2 \times 10^2\ s^{-1}$. Since DNA site concentration is 0.19 mM, the calculated second-order rate constant is $1.05 \times 10^6\ M^{-1}\ s^{-1}$, in excellent agreement with the value found from the dissociation experiments.

Discussion

(1) Minimal Scheme for Binding and Exchange. In order to analyze the H–D exchange data, it is necessary to refer to some model of the drug binding reaction as it mediates H–D exchange in the free and bound states. Direct kinetic experiments imply that the minimal scheme (eq 2) adequately accounts for the association of drug to DNA sites in the presence of high salt. Accordingly, H–D exchange from the

system consisting of free and bound EB can be represented as follows:

$$\begin{array}{ccc} \mathrm{DNA(EB_D)} & \underset{k_{+1}}{\overset{k_{-1}}{\rightleftharpoons}} & \mathrm{DNA + EB_D} \\ \Big\downarrow k_b & & \Big\downarrow k_f \\ \mathrm{DNA(EB_H)} & & \mathrm{DNA + EB_H} \end{array} \qquad (4)$$

Here subscripts D and H refer to deuterated and protonated forms of the dye molecule, k_b denotes the exchange rate of EB hydrogens from the complex, and k_f denotes that from free EB. As in eq 3, k_{-1} and k_{+1} denote the off and on rates for drug to DNA sites, with K representing the association constant.

This scheme has been analyzed previously in detail by Schreier & Baldwin (1976) using the steady-state assumption. Equation 5a represents their result, written in the notation of eq 4.

$$k_{ex} = \frac{k_{-1}k_f + k_b(k_{+1}[\mathrm{DNA}] + k_f)}{k_{-1} + k_{+1}[\mathrm{DNA}] + k_f} \qquad (5a)$$

Here DNA refers to the concentration of *free* DNA sites (5 P/site). This equation includes two special cases of interest here. If the on rate of drug is much faster than H exchange so that $k_{+1}[\mathrm{DNA}] \gg k_f$, the exchange rate consists of additive contributions from free and bound drug populations:

$$k_{ex} = \frac{k_f}{1 + K[\mathrm{DNA}]} + \frac{k_b K[\mathrm{DNA}]}{1 + K[\mathrm{DNA}]} = fk_f + (1-f)k_b \qquad (5b)$$

where f denotes the fraction of free EB and $1-f$ is the fraction bound. This is as one expects given eq 4. On the other hand, when the H exchange rate of free dye exceeds both the drug on rate and the drug off rate, eq 5a reduces to

$$k_{ex} = k_{-1} + k_b \qquad (5c)$$

These expressions permit us to determine each of the rate constants in eq 4 unambiguously.

(*2*) *Analysis for Equilibrium Binding Constant.* First, we apply eq 5a to the data of Figure 5. The equilibrium constant K can be evaluated from the relationships

$$K = \frac{[\mathrm{EB{\cdot}DNA}]}{[\mathrm{EB}]_f[\mathrm{DNA}]_f} = \frac{1-f}{f[[\mathrm{DNA}]_T - [\mathrm{EB}]_T(1-f)]} \qquad (6a)$$

$$f = \frac{k_{ex} - k_b}{k_f - k_b} = \frac{[\mathrm{EB}]_f}{[\mathrm{EB}]_T} \qquad (6b)$$

Here $[\mathrm{EB}]_f$ is the free drug concentration and $[\mathrm{EB}]_T$ is the total drug concentration. Equation 6a arises from the definition of K, and eq 6b by rearrangement of eq 5b.

First, we observe that one cannot obtain valid estimates of K from the apparent titration of drug on the left arm of the curve in Figure 4, because the situation represents one in which [EB] initially is well above the dissociation constant. However, the dependence of k_{ex} on DNA concentration permits one to estimate the effective site size independently of any other measurement. The data in Table II are consistent with an initial utilization of DNA corresponding to a site size of 4.5 phosphates/bound ethidium, in agreement with the value derived from the direct stopped-flow experiments. This value assumes that the exchange rate of bound ethidium is zero.

Second, as the free ethidium concentration approaches the plateau on the right of Figure 4, values of K can be determined with some precision. There are two extreme assumptions we can make concerning this region of the curve: (1) $k_b = 0.2$ s^{-1}, so that this limiting exchange rate is due to exchange from the bound state, and (2) $k_b = 0$, in which case the limiting rate is due to exchange from the residual free ethidium. The equilibrium constants estimated for these two cases are tabulated in Table II, which shows that the difference in estimated K values is roughly a factor of 2 in the region of concentration for which both can be validly obtained. Assuming $k_b = 0.2$ s^{-1} makes it impossible to calculate the equilibrium constant at high DNA concentrations. However, in the other case valid estimates can be made even from this region of Figure 4, as is indicated in Table II. If, as in the case of protein–protein complexes, the value of $k_b = 0$ can be applied, the method can be seen to make it possible to estimate K under conditions well above the dissociation constant (Schreier & Baldwin, 1976). In the present case, the uncertainty introduced into the estimated K value is moderate: for case 1, $\bar{K} = (6.2 \pm 0.6) \times 10^4$ M^{-1}; for case 2, $\bar{K} = (2.5 \pm 1) \times 10^4$. Hence, in any case we have determined the binding constant K as $(5 \pm 3) \times 10^4$ (at pH 9). Relatively few points of the data in Figure 4 permit estimation of a valid K. The correction of these values for site exclusion according to McGhee & von Hippel (1974) results in a negligible difference from these values. LePecq & Paoletti (1967) reported a K value of 8×10^4 M^{-1} from binding experiments based on fluorescence enhancement measurements under similar conditions. A plot of k_{ex} vs. f according to eq 5b is linear as required.

(*3*) *Rate Constants.* In the presence of phosphate catalysis (Figure 5), k_f increases to the point that conditions approximating eq 5c are attained. The asymptotic exchange rate measured in Figure 5 thus reveals the dissociation rate of the complex, and this yields $k_{-1} = 32$ s^{-1} (pH 6.5) and $k_{-1} = 27$ s^{-1} (pH 7.5).

Examination of Figure 2 indicates that the specific acid-catalyzed rates are already approaching the on rate of ethidium even in the absence of phosphate. Hence, one does not obtain a simple decade increase in the exchange rates corresponding to [P] = 0 in Figure 5 between pH 7.5 and pH 6.5. It should be noted that the titrations in Figure 5 were started in the presence of 1 mM phosphate to stabilize pH; the points designated [P] = 0 include this amount of additional catalysis.

The dependence of k_{ex} on k_f before the asymptotic limit is reached permits one to test eq 5 more stringently. Under conditions where ethidium is predominantly bound, that is, when $k_{+1}[\mathrm{DNA}] \gg k_{-1}$, eq 5a takes on a simple Michaelian form:

$$k_{ex} = k_b + \frac{k_{-1}k_f}{k_f + k_{+1}[\mathrm{DNA}]} \qquad (7a)$$

Hence, the reciprocal plot

$$\frac{1}{k_{ex} - k_b} = \frac{1}{k_{-1}} + \frac{K[\mathrm{DNA}]}{k_f} \qquad (7b)$$

should be linear. The rate constant for exchange of free ethidium, k_f, is proportional to the concentration of phosphate (eq 1 and Figure 3). Thus, the linearity of the insert to Figure 5 provides a stringent test of the underlying scheme (eq 4) and again indicates the self-consistency of the model and the rate constants measured.

The rate constants k_{-1} and k_{+1} determined from H–D exchange (Table II) and by direct kinetic analysis are summarized in Table III. It is concluded that H–D exchange provides a usable probe of the dynamics of this ligand–macromolecule binding process. In high salt (1.0 M NaCl), binding of EB yields a single apparent complex with on rate $k_{+1} = 10^6$ M^{-1} s^{-1} and $k_{-1} = 30$ s^{-1} at 20 °C.

The secondary binding modes available to ethidium in low salt are suppressed by the high salt conditions used here [see

Table III: Apparent Kinetic and Equilibrium Constants for Ethidium Binding to DNA

	direct mixing experiments	dilution "jump" experiment	H-D exchange
k_{+1} (M^{-1} s^{-1})	1.0×10^6 (pH 6.5)	1.0×10^6	$(2 \times 10^6)^a$
k_{-1} (s^{-1})		32 (pH 6.5)	32 (pH 6.5) 27 (pH 7.5)
K (M)		3.2×10^4 (pH 6.5)	6.6×10^4 [b] (pH 9)

[a] Value obtained from k_{-1} and K measured by H-D exchange at different pH values. [b] Fluorescence enhancement measurements indicate slightly higher binding at pH 9 than at pH 7 (LePecq & Paoletti, 1967).

Bresloff & Crothers (1975)]. Neither technique we have used detects the direct ethidium–DNA transfer process reported by Bresloff & Crothers (1975) using temperature-jump relaxation experiments. This process could well escape detection by our measurements and cannot be excluded. The significant feature for this discussion is that no intermediates that affect the exchange of ethidium are implicated by our data. In general, this might not be the case, and exchange could be responsive to different characteristics of the interaction than those revealed by direct binding analysis.

(*4*) *Structural and Dynamic Implications.* How does the requisite 3.4-Å vacancy between adjacent base pairs arise? We have previously reported from H–D exchange measurements that base-pair separation at internal pairs of a duplex is a slow but moderately favorable reaction; for example, H exchange rate at 20 °C is ~ 1 s^{-1} for poly[r(A·U)] and the opening equilibrium constant close to 0.05 (Teitelbaum & Englander, 1975a,b; Mandal et al., 1979). It seems now that DNA behaves similarly, but that G-C base pairs are a factor of 10 less open. Under some of the experimental conditions in Figure 6, the pseudo-first-order rate for binding ethidium to DNA "sites" is in excess of 20 s^{-1}. The equilibrium constant for a presumed prior opening reaction would be $\sim 10^{-3}$, since the k_{+1} is this factor below the diffusion-limited rate. Thus, coupling between intercalation of ethidium and breaking of base-pair H bonds cannot be excluded. Li & Crothers (1969) concluded that the two processes are not coupled in the case of proflavin–DNA binding. A second possibility is that intercalation occurs via preexisting bending or twisting and accordion modes of the double helix (Gabbay et al., 1973; Barkley & Zimm, 1979). The different possibilities can be investigated by studies on intercalation at varying drug concentrations and temperatures, and we will report on this subsequently.

The exchange data focus on the state of the amino groups in ethidium as it is complexed with DNA. In the complex, the H–D exchange rate is slower than in the free dye by at least 18-fold, and perhaps much more. The observed retardation raises the question of whether or not the amino groups of ethidium are hydrogen bonded. Structural studies on crystalline oligonucleotide complexes of ethidium with dinucleotides (Tsai et al., 1975) do not specify a hydrogen bond between these NH_2 groups and the phosphate oxygens of the backbone that lie nearest to these groups, though reasonable hydrogen bonds extending between these groups can be accommodated in the kind of models determined from crystal structures. While we feel that H bonding probably is the main reason for retardation, we cannot rigorously prove from the exchange data here that H bonds are present in solution. Moderate retardation in exchange might be interpreted on more general steric grounds. Phosphate catalysis of the bound form of ethidium is slower by a factor of at least 50 than that for the free state as can be calculated by comparing the data in Figure 5 with the acid phosphate effect at pH 7.5 on free ethidium. This situation could arise whether or not the amino groups are hydrogen bonded within the complex, since on steric grounds alone it would be difficult for phosphate to catalyze the bound drug. If exchange proceeds via the likely tetrahedral C–NH_3^+ intermediate and this structure is not readily accommodated within the duplex, this would in itself inhibit the exchange process. Electrostatic repulsion of phosphate from the DNA duplex could also contribute to ineffective catalysis of the complex. It is nonetheless worth noting that retardation by intrinsic factors of 50 or more generally corresponds to situations involving hydrogen bonding.

(*5*) *Methodology.* The utility of the spectrally detected H–D exchange process as a probe of ligand interactions should be underscored. This is because the measurement registers the summed dissociation events that have occurred since the reaction started (from time of mixing). One advantage of using H exchange rather than direct analysis of the association itself is that concentrations well above the dissociation constant can be used. This is valuable for study of extremely tight binding processes, as has been pointed out by Schreier & Baldwin (1976), who used exchange of ^3H from the S-peptide of ribonuclease to determine parameters of the tight binding between S-peptide and RNase S-protein [see also Barksdale & Rosenberg (1978)]. The use of the spectral characteristics of the ligand as described in this work allows a considerable simplification in experimental procedure and extends these measurements into the stopped-flow scale. Thus, binding interactions which could not be dealt with before, including dye–nucleic acid and nucleotide–protein systems, can now be conveniently studied.

One further point should be noted in connection with the use of the stopped-flow instrumentation we have described here that does not apply to other exchange measurements. This concerns the possible effect of ligand reequilibration following the dilution of the sample in D_2O into H_2O. In conditions such as we have used in the ethidium–DNA experiments, where the complex is at concentrations above the dissociation constant before and after dilution, reequilibration will not be a factor. This means that in cases of extremely tight binding the stopped-flow procedure should apply.

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References

Barkley, M. D., & Zimm, B. H. (1979) *J. Chem. Phys. 70*, 2991–3007.

Barksdale, A. D., & Rosenberg, A. (1978) *Methods Enzymol. 48*, 321–346.

Bernasconi, C. F. (1976) *Relaxation Kinetics*, Academic Press, New York.

Bresloff, J. L., & Crothers, D. M. (1975) *J. Mol. Biol. 95*, 103–123.

Cross, D. G. (1975) *Biochemistry 14*, 357–362.

Englander, J. J., Kallenbach, N. R., & Englander, S. W. (1972) *J. Mol. Biol. 63*, 153–169.

Englander, J. J., Calhoun, D. B., & Englander, S. W. (1979) *Anal. Biochem. 92*, 517–524.

Gabbay, E. J., Destefano, R., & Baxter, C. S. (1973) *Biochem. Biophys. Res. Commun. 51*, 1083–1089.

Hanson, C. V. (1971) *J. Mol. Biol. 58*, 847–863.

LePecq, J. B., & Paoletti, C. (1967) *J. Mol. Biol. 27*, 87–106.
Li, H. J., & Crothers, D. M. (1969) *J. Mol. Biol. 36*, 461–477.
Mandal, C., Kallenbach, N. R., & Englander, S. W. (1979) *J. Mol. Biol. 135*, 391–411.
McGhee, J. D., & von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469–489.
Nakanishi, M., & Tsuboi, M. (1978a) *J. Mol. Biol. 124*, 61–71.
Nakanishi, M., & Tsuboi, M. (1978b) *J. Am. Chem. Soc. 100*, 1273–1275.
Neidle, S. (1979) *Prog. Med. Chem. 16*, 151–221.
Schreier, A. A., & Baldwin, R. L. (1976) *J. Mol. Biol. 105*, 409–426.
Takahashi, T., Nakanishi, M., & Tsuboi, M. (1978) *Bull. Chem. Soc. Jpn. 51*, 1988–1990.
Teitelbaum, H., & Englander, S. W. (1975a) *J. Mol. Biol. 92*, 55–78.
Teitelbaum, H., & Englander, S. W. (1975b) *J. Mol. Biol. 92*, 79–92.
Tsai, C. C., Jain, S. C., & Sobell, H. M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 628–632.
Waring, M. J. (1965) *J. Mol. Biol. 13*, 269–282.

Effects of Ring Substituents and Linker Chains on the Bifunctional Intercalation of Diacridines into Deoxyribonucleic Acid†

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ABSTRACT: Sedimentation experiments with closed circular duplex PM2 deoxyribonucleic acid (DNA) and viscosity measurements with sonicated rodlike DNA fragments have been performed to investigate unwinding and extension of the DNA helix associated with binding of homologous series of diacridines. When the acridine nuclei are linked via 9-amino substituents with chains permitting interchromophore separation in the critical region between 8.8 and 11.3 Å, it is found that substituents at positions 2, 3, and 6 restrict bifunctional intercalation to compounds capable of sandwiching at least two base pairs in the intercalated complex. Unsubstituted diacridines, and derivatives bearing an ethyl group at position 4, can bis-intercalate with a significantly shorter linking chain. The presence of amino groups in the linker does not affect the ability to bis-intercalate but increases the helix unwinding angle by 30–40% and decreases the helix extension per bound ligand molecule by 11–18%. Bifunctional reaction is observed with a diacridine linked via the 4–4′ positions showing that 9-aminoacridine can intercalate in at least two major orientations with the 9 or 10 position directed toward the helix axis. Both orientations can be accommodated simultaneously within a single diacridine linked via the 9–4′ positions with a bridge sufficiently long to encompass two base pairs. An acridone substituted at positions 4 and 5 with positively charged side chains intercalates with a helix unwinding angle indistinguishable from that of ethidium whereas an uncharged bisacridone failed to yield a detectable complex with DNA. Tilorone dihydrochloride, a structurally similar antiviral drug, was found to intercalate with an unwinding angle approximately half that of ethidium. The potentially bifunctional bisquinoline drug dequalinium causes relaxation of the supercoiling of PM2 DNA but probably not by simultaneous intercalation of both its chromophores.

Efforts to develop potential chemotherapeutic DNA-binding drugs endowed with enhanced selective toxicity center largely on studies of polyfunctional intercalating agents (Waring & Wakelin, 1974; Le Pecq et al., 1975; Canellakis et al., 1976; Cain et al., 1978; Gaugain et al., 1978; Kuhlmann et al., 1978; Wakelin et al., 1978). The underlying principle is that bis-intercalation affords the opportunity for improving both nucleotide sequence selectivity and specificity for DNA per se as a result of the larger binding constants expected for drugs of this type. Recently, it has been demonstrated that the transition from mono- to bifunctional reaction in an homologous series of diacridines is indeed accompanied by a substantial increase in the binding constant (Wakelin et al., 1979).

LePecq et al. (1975) studied a series of diacridines derived from 6-chloro-2-methoxyacridine linked through the 9 position via various polyamines. They concluded that bifunctional reaction only occurs when the linkage is sufficiently long to permit two base pairs to be accommodated between the chromophores. Similarly, it is likely that the rigid cyclic peptide which separates the quinoxaline chromophores in echinomycin by 10.2 Å restricts this antibiotic to binding via a "two-base-pair sandwich" model (Waring & Wakelin, 1974; Wakelin & Waring, 1976; Ughetto & Waring, 1977; Cheung et al., 1978). In contrast to these two examples, Wakelin et al. (1976, 1978) showed that in a series of unsubstituted diacridines linked via a simple methylene chain bis-intercalation is first observed when the linkage is long enough to span only one base pair, assuming a Fuller–Waring-type geometry for the intercalated complex (Fuller & Waring, 1964). Kuhlmann et al. (1978) reported bifunctional reaction for a dimer of ethidium linked through the para position of the 6-phenyl ring, whereas Gaugain et al. (1978) failed to observe bis-intercalation with an ethidium–acridine dimer and a simple ethidium dimer, both of which were linked through the quaternary ring nitrogen.

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