Mechanism of Intercalation into the DNA Double Helix by Ethidium

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ABSTRACT: The mechanism of intercalation into DNA double helices by ethidium has been analyzed by temperature-jump relaxation and stopped-flow measurements using fluorescence detection. Artifacts due to field- or flow-induced alignment have been eliminated by measurements under magic angle conditions; the theoretical basis for suppression of orientation effects resulting from external forces is given for the case of fluorescence measurements. Excluded site effects have been avoided by restriction to low degrees of binding. The temperature-jump relaxation observed for ethidium binding to DNA could be described by single exponentials under most conditions. The reciprocal time constants increased linearly with the DNA concentration, leading to association rate constants of 2.7×10^6 M^{-1} s^{-1} at 12 °C. These rate constants are virtually independent of the DNA chain length for samples with 200, 500, 4228, and 30 000 base pairs, showing that the rate is controlled by reaction and not by a diffusive process. At high DNA concentrations around 200 μM, an additional relaxation effect with an amplitude opposite to the main one is observed which is probably due to some conformational change of the DNA–ethidium complex. The results obtained by stopped-flow measurements are consistent with those from T-jump measurements, but owing to higher amplitudes and better signal to noise ratios, the stopped-flow data clearly require two exponentials for satisfactory representation. The reciprocal time constants for both processes increase linearly with the DNA concentration. The simplest mechanism consistent with this result involves parallel formation of two different complexes with a direct transfer of ethidium between the binding sites. The experimental data for a synthetic DNA with one type of base pairs, poly[d(A–T)], are very similar to those for natural DNA; thus, the relatively complex reaction mechanism of intercalation is not due to base-pair heterogeneity. Apparently the two complexes are formed by intercalation of ethidium between the base pairs from the directions of the major and the minor grooves of the double helix. Our results demonstrate that the Joule temperature-jump technique does not introduce artifacts due to field-induced processes, when this technique is used with appropriate care.

Although the intercalation mechanism of aromatic residues into the DNA double helix has been investigated many times and by various techniques, the conclusions on this reaction are still widely divergent (Magde et al., 1974; Bresloff & Crothers, 1975; Jovin & Striker, 1977; Wakelin & Waring, 1980; Mandal et al., 1980; Ramstein et al., 1980; Feigon et al., 1982; Ryan & Crothers, 1984; Wilson et al., 1985; Macgregor et al., 1985, 1987). Some authors have found a very simple reaction mechanism with a single reaction step, whereas others have presented complex mechanisms with up to four reaction steps. The conclusions on the main reaction step are also contradictory: according to several investigations the intercalation is a reaction-controlled process, but according to recent studies of Macgregor et al. (1987) "intercalation is controlled by a diffusive process". Macgregor et al. conclude that the rate of intercalation is determined both by diffusion and by the probability of a conformational change of base pairs, which is required for intercalation. Some of the differences may be explained by the fact that the reaction is influenced by many different parameters, which are very exactly equivalent in the various investigations. Nevertheless, the essentials of the intercalation reaction should remain constant in spite of some differences in the conditions. Thus, it has been suspected that the divergence mainly results from problems associated with some of the techniques used for investigation. Some authors (Marcandalli et al., 1984; Macgregor et al., 1985) suspect that the Joule temperature-jump technique is the main source of the difficulties. According to these authors, relaxation curves obtained by this technique cannot be taken as clean evidence because of perturbations resulting from the electric field used for heating. Marcandalli et al. (1984) even conclude that "the many artifacts introduced by this technique and its severe limitations completely obscure the process under investigation and no reliable information can be obtained, if such complex biological systems as the double helix of DNA are investigated". Should we forget all of the data collected by the Joule temperature-jump method on "complex biological systems" in general and on DNA double helices in particular?

The issue is of sufficiently general interest; thus, we have started a comparative investigation of the intercalation of ethidium into the DNA double helix by Joule temperature-jump and stopped-flow techniques. Special attention was given to artifacts, which may arise from the application of electric field pulses. Our results demonstrate that artifacts can be easily avoided using procedures which were described in the literature many years ago (Labhart, 1961; Dourlent et al., 1974; Porschke, 1974) and which have been routinely used in this laboratory since then. We have also checked the recent results obtained by pressure-jump measurements which suggest that the "intercalation is controlled by a diffusive process" (Macgregor et al., 1987). Our measurements on DNA samples over a wide range of chain lengths show that intercalation is a process controlled by reaction and not by a diffusive process.

MATERIALS AND METHODS

Ethidium bromide was obtained from Merck (Darmstadt) and was used without further purification. The concentrations of stock solutions were evaluated from measurements of the absorbance at 480 nm using an extinction coefficient of 5700 M^{-1} cm^{-1}.

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DNA from calf thymus with a chain length of approximately 30 000 base pairs was obtained from Boehringer Mannheim. Part of this sample was sonicated to an average chain length of 500 base pairs. Another part of this sample was digested with *Hae*III restriction nuclease; the resulting fragments were separated by column chromatography on Sepharose. For some of the experiments described below we used a fraction from the *Hae*III digest with an average chain length of 200 base pairs. The average chain lengths were determined by acrylamide gel electrophoresis using a set of well-defined restriction fragments as reference.

Another DNA sample was prepared from a bacterial strain carrying the plasmid DNA pRW574 with 4228 base pairs. The plasmid DNA was purified by standard procedures and proved to be of homogeneous chain length according to gel electrophoresis; however, this DNA was a mixture of superhelical, closed-circular, and open-circular forms. For comparison we also used a poly[d(A–T)] sample from Boehringer Mannheim with an average chain length of 10 000 base pairs according to gel electrophoresis. Unless specified otherwise, concentrations of DNA solutions are given in units of moles of base pairs per dm^3.

All DNA samples were dialyzed extensively according to the following procedure: in the first stage the samples were dialyzed against 1 M NaCl, 1 mM sodium cacodylate (pH 7), and 1 mM EDTA. In the final stage the samples were dialyzed against the standard buffer used for the measurements, 0.1 M $NaClO_4$, 10 mM sodium cacodylate (pH 7.0), and 0.25 mM EDTA.

Equilibrium constants for the binding of ethidium to DNA were determined by standard fluorescence titrations using an SLM 8000 spectrofluorimeter. The data were corrected for inner filter effects using a model described by Porschke and Rauh (1983) and were evaluated according to the excluded site binding model (McGhee & von Hippel, 1974).

For the temperature-jump measurements we used an instrument constructed in this institute. We used a 600-W mercury–xenon lamp together with a Schoeffel GM250 monochromator as light source. Usually the fluorescence was excited at 313 nm and collected behind 530-nm cutoff filters, GG530 from Schott (Mainz). The instrument used for our stopped-flow measurements was also constructed in this institute. The "dead" time of this instrument was determined by the reaction of *N*-bromosuccinimide with *N*-acetyltryptophanamide according to Peterman (1979), and was found to be about 1 ms in the standard configuration (cf. below). The experimental data were transiently stored on a Biomation 1010, transferred to a PC, and finally transmitted to the facilities of the Gesellschaft für wissenschaftliche Datenverarbeitung mbH Göttingen for fitting by exponentials according to various procedures.

TEMPERATURE-JUMP EXPERIMENTS

Dichroism of the Fluorescence. As shown in the supplementary material, effects due to field-induced alignment can be avoided by measurements at magic angle conditions. For verification of our magic angle conditions, we have used DNA–ethidium complexes in solutions of 1.1 mM salt concentration, where field-induced orientation effects are much larger than those found at salt concentrations around 0.1 M. Using an electric field jump instrument with an optical system adapted for measurements with polarized light, we have checked our T-jump cell and found that the very large dichroism of the fluoresence disappeared completely when both excitation and emission light paths were adjusted to the magic angle.

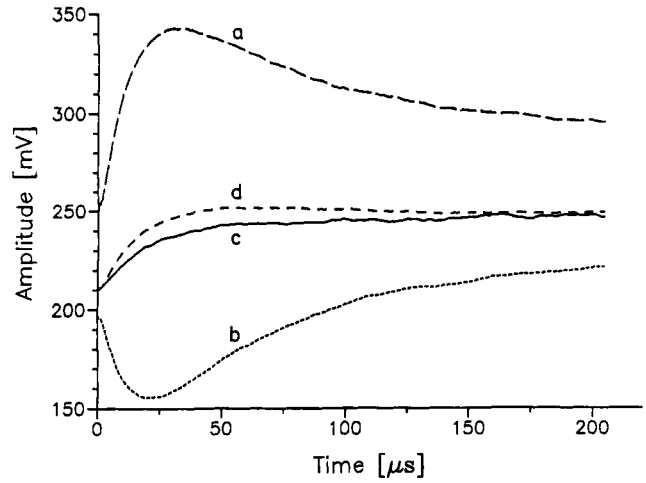

FIGURE 1: Fast part of the temperature-jump relaxation detected by fluorescence for 50 μM KT-DNA (30 kbp) and 3.33 μM ethidium recorded with different directions of polarizers in excitation and emission with respect to the transient electric field: (a) parallel; (b) perpendicular; (c) magic angle (cf. text); (d) without polarizers (light used for excitation partly polarized, e.g., due to the grating monochromator, but degree of polarization not quantified). The amplitude of the curves at $t = 0$ is arbitrary; curves c and d are superimposed at $t = 0$. The slightly sigmodial shape at the start of the curves results from convolution of the chemical/physical relaxation with the process of heating (heating time constant, 1.4 μs; rise time of the detection circuit, 21.6 μs; temperature jump, 2.9 °C; final temperature, 24 °C).

Corresponding experiments with our temperature-jump instrument showed that the dichroism of the fluorescence also was reduced considerably at magic angle conditions, but not completely. The obvious reason for the remaining effect was the optical system for collection of the fluorescent light, which is constructed using lenses with a large aperture in order to collect as much light as possible. Due to the large aperture, the magic angle conditions are not satisfied exactly. We found that under these conditions the dichroism was suppressed completely by adjustment of the polarizers used in the emission light beam to angles of about 48° instead of 55° (cf. Figure 1).

We have used this adjustment of the polarizers for measurements of the temperature-jump relaxation in the standard buffer. First, we compared relaxation time constants measured in the presence and in the absence of the polarizers. For this comparison we used the sonicated DNA sample with ~500 bp. In the range of DNA concentrations c_d from 2.6 to 250 μM and ethidium concentrations c_e defined by the ratio $c_d/c_e = 15$, the relaxation curves could be fit by single exponentials at a satisfactory accuracy. A difference between the time constants τ in the presence and in the absence of the polarizers was found only at high reactant concentrations ($c_d > 100$ μM), where the τ values are below 1 ms. As expected, the orientation effects, induced during the discharge of the capacitor, decay within a time determined by the time constants of the capacitor discharge and the rotational diffusion of the DNA fragments. According to these results, we have used the polarizers for all measurements with the sonicated DNA sample, when the relaxation time constant was below 1 ms. Furthermore, we used the polarizers for all measurements with the 30-kbp DNA sample, because its rotational correlation time constants extend to the seconds time range. The polarizers were not used for the other measurements, because the signal to noise ratio is reduced in the presence of the polarizers due to their absorbance of light.

Main Relaxation Process Showing a Reaction-Controlled Mechanism. As already mentioned above, the fluorescence-

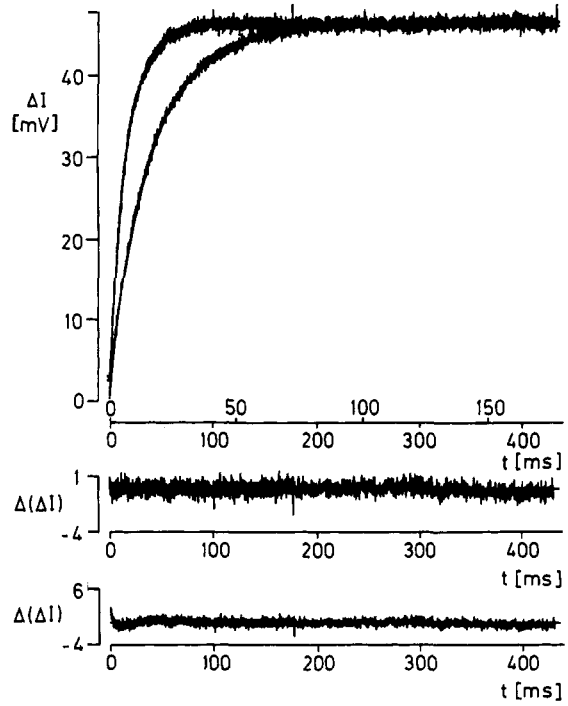

FIGURE 2: Temperature-jump relaxation detected by fluorescence for 20 μM KT-DNA 500 bp and 1.32 μM ethidium at 12 °C at two different time scales (average of six individual jumps, $\Delta T = 2.9$ °C). The lower panels show the residuals of fits with two exponentials ($\tau_1 \approx 8$ ms, $\tau_2 \approx 20$ ms) and with one exponential ($\tau = 17.3$ ms).

detected temperature-jump relaxation can be fit by single exponentials at a satisfactory accuracy (cf. Figure 2). In order to avoid complications due to excluded site effects, the measurements were restricted to low binding densities by using a ratio of the DNA concentration c_d (given in units of base pairs) to the ethidium concentration c_e of $c_d/c_e = 15$. Thus, the maximal degree of binding is 13.3% (with the "exclusion" of 2 base pairs), and we may expect a pseudo-first-order reaction for the association of DNA and ethidium. We have checked that the time constants observed in this range do not depend on the degree of binding: the τ values are defined by the reactant concentration, which is equivalent to the concentration of free DNA binding sites at our fixed c_d/c_e ratio.

Measurements over the range of concentrations, where the amplitudes are sufficiently large, show a simple linear dependence of the reciprocal time constant on the reactant concentration (cf. Figure 3) according to

$$\text{DNA} + \text{ethidium} \underset{k^-}{\overset{k^+}{\rightleftharpoons}} \text{complex} \qquad (1)$$

and

$$1/\tau = k^+(\bar{c}_b + \bar{c}_e) + k^- \qquad (2)$$

where k^+ and k^- are the rate constants of association and dissociation, respectively; $\bar{c}_b$ and $\bar{c}_e$ denote the free concentrations of binding sites on the DNA and of ethidium, respectively.

The association rate constant derived from the measurements on the 500-bp DNA at 12 °C is 2.7×10^6 M^{-1} s^{-1}; thus, the reaction can hardly be assigned as diffusion-controlled. In spite of the low overall reaction rate, which has been found by various authors including Macgregor et al. (1987), Macgregor et al. concluded from their investigations that intercalation of ethidium into double-helical DNA is "controlled by a diffusive process". We have analyzed this problem

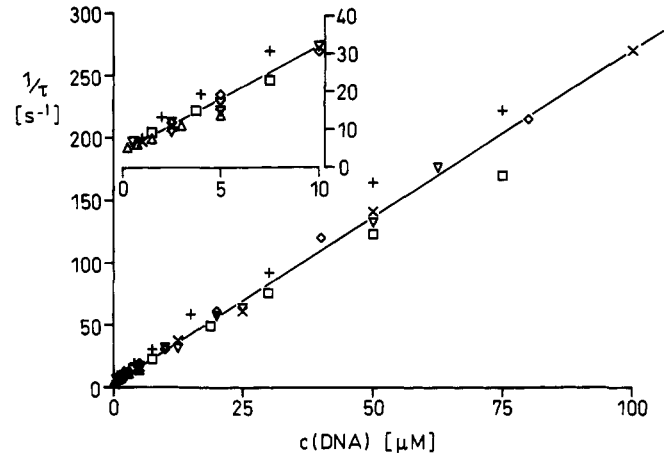

FIGURE 3: Reciprocal time constants $1/\tau$ obtained from single exponential fits of the DNA–ethidium relaxation as a function of the DNA concentration in units of base pairs from temperature-jump experiments (×, 30 kbp; +, 4 kbp; ▽, 500 bp; ◇, 200 bp) and from stopped-flow experiments (△, 30 kbp; □ 500 bp) at 12 °C.

by measurements with DNA samples of widely different molecular weights. In all cases we have measured the temperature-jump relaxation for the different samples at given concentrations, which were defined on the basis of base pairs for the DNA and the ratio $c_d/c_e = 15$ for ethidium. At a given DNA residue concentration, the concentration in terms of polymer molecules is much lower for samples with a high chain length N than for samples with low N. Thus, an increase of N should lead to an increase of the τ value, if the reaction is controlled by diffusion. As shown in Figure 3, the relaxation time constants do not change in the range of chain lengths from 200 to 30 000 bp. These results are not consistent with a diffusion-controlled reaction, but indicate a reaction-controlled process: the rate of the process associated with the large increase in the fluorescence intensity is determined by a reaction like the insertion of the aromatic residue into the DNA, but not by the diffusion of ethidium to the DNA.

The observed chain length dependence of the reaction rate has been compared with the predictions of pertinent theories. For this purpose, we present the experimental rate constants relative to that measured for the DNA with 200 bp. As shown in Figure 4, the observed rates are in exact agreement with the prediction for the reaction-controlled process and are not consistent with the predictions for a diffusion-controlled reaction according to models for a "coiled polymer sphere" and for a "solid polymer sphere" (Porschke, 1979; Berg et al., 1981; Lohman, 1985). For all polymer models the chain length dependence for a diffusion-controlled reaction mainly results from the fact that the reaction radius of long polymer chains increases with the square root of the chain length. The chain length dependence expected according to theory has been observed for the binding reaction of simple basic peptides to polynucleotides (Porschke, 1978, 1979); the first version of a polymer sphere model for rate constants of ligand binding (Porschke, 1979) was developed for the interpretation of these data. The expected chain length dependence is assumed to hold for chain lengths ≥200 base pairs in the plot according to the solid polymer sphere model shown in Figure 4. In the case of the coiled polymer sphere model, the possibility is included that ligands diffuse through the polymer sphere without reaction. In the limit of low chain lengths, the coiled polymer sphere model provides a chain length dependence which is consistent with that expected according to rod models. A detailed review of these models has been given by Lohman (1985). The rate constants evaluated for different DNA

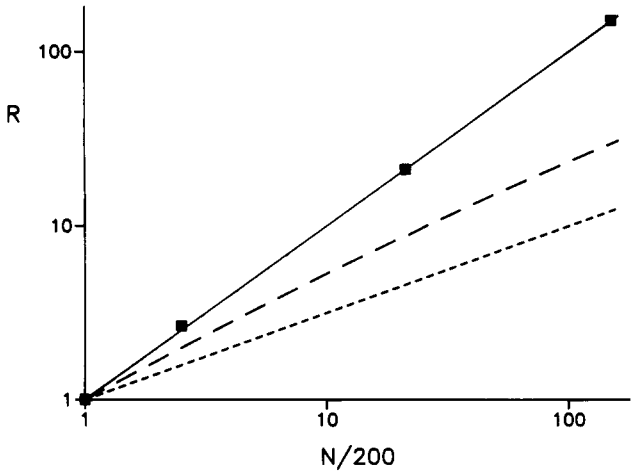

FIGURE 4: Relative association rate constant R as a function of the relative chain length $N/200$. We use the result obtained for the 200-bp sample as a reference: the rate constants for the DNA samples of different chain lengths are calculated in units of (M helices)$^{-1}$ (s)$^{-1}$ and are given relative to the value obtained for the sample with 200 bp. The data exactly match the prediction for the reaction-controlled model (—) and are not consistent with diffusion-controlled mechanisms according to the coiled and solid sphere models – – and - - -, respectively). The line for the solid sphere model is calculated under the assumption that the rate increases with the square root of the chain length. The line for the coiled sphere model is calculated according to eq 5.3 of Berg et al. (1981) with the following parameters: persistence length, 44 nm; radius of the double helix, 1.5 nm; rise of the helix axis per base pair, 0.34 nm.

samples and for various conditions according to the simple one-step reaction model are compiled in Table I.

Evidence for Additional Reaction Steps. After assignment of the main relaxation effect to a reaction-controlled process, we have to postulate an additional relaxation process reflecting the preceding reaction step corresponding to the formation of a first contact between the reactants. This process is expected to be relatively fast. The relaxation curves detected by fluorescence measurements show a large amplitude with time constant(s) below a few microseconds. However, a fast relaxation process is virtually always observed in fluorescence-detected T-jump relaxation curves, because in general fluorescence intensities decrease with increasing temperature due to increasing quenching rates. The postulated chemical relaxation effect may be hidden within the large physical effect. We have tried to identify such a hidden effect by measurements with particularly high time resolution and by application of deconvolution techniques. Some evidence has been obtained for a chemical relaxation effect with a time constant of about 0.5 μs, but its amplitude was not large enough for an unequivocal identification.

As described above, the main relaxation effect found by the temperature-jump technique in the millisecond time range could be fit by single exponentials at a satisfactory accuracy. At low concentrations, however, the quality of most fits could be improved by admitting a second exponential. The sum of residuals was only slightly decreased upon admitting a second exponential, and the time constants of the two-exponential fits did not differ by more than a factor of 3. Thus, the time constants from the two-exponential fits were not sufficiently well defined and could hardly be used as a basis for further evaluations. The stopped-flow data discussed below are more accurate in this respect because of higher amplitudes, and they demonstrate the existence of more than a single relaxation process in the millisecond time range. Because some distribution of time constants may be expected for natural DNA due to a heterogeneity of binding sites resulting from sequence heterogeneity, we have investigated ethidium binding to the homogeneous poly[d(A–T)] and observed relaxation curves very similar to those found for natural DNA. This issue will be discussed in more detail below.

While the T-jump technique is not sensitive enough for an unequivocal characterization of the relaxation heterogeneity in the millisecond time range, it reveals another process which has not been detected by our stopped-flow measurements. The additional process is observed at high DNA concentrations above 50 μM, with a time constant of 10–50 ms, and is characterized by an amplitude opposite to that of the main relaxation effect (cf. Figure 5). This process has been observed both for natural DNA and for poly[d(A–T)]. Because the amplitude of the additional effect is relatively small ($\Delta I \leq 0.05\%$), it has not been investigated in detail. Apparently it is due to some conformational change which is induced by temperature jumps and, thus, is not visible in stopped-flow experiments.

STOPPED-FLOW EXPERIMENTS

Because we have used the magic angle technique, the results of our T-jump relaxation studies cannot be perturbed by any field-induced orientation effects. Nevertheless, we wanted to present an independent proof of the validity of our results by a different technique. For this purpose we have characterized the ethidium intercalation reaction by stopped-flow measurements. Although the samples clearly are not exposed to electric fields during stopped-flow measurements, nevertheless there can be orientation effects of polymer samples induced by the flow. Because the time resolution of standard stopped-flow instruments is limited to about 1 ms, orientation effects cannot contribute to the measured signals for samples with rotational time constants much below 1 ms. Thus, the stopped-flow experiments for our samples with 500 bp did not require any precautions: its largest rotational time constant is in the range of 25 μs (at 20 °C). For the long DNA sample with 30 kbp, however, we again had to use the magic angle technique, because the rotational time constants of this sample extend to the seconds time range. Furthermore, we had to protect this sample against degradation by shear gradients by using reduced flow rates. We reduced the flow rate in this case by simple introduction of a narrow channel *behind* the observation chamber. The diameter of this channel was reduced until degradation of the 30-kbp DNA was no longer detected by agarose gel electrophoresis of samples taken from the observation chamber (cf. Porschke (1984)). Obviously, the reduced flow rate leads to a decrease of the limit time resolution of the stopped-flow instrument; thus, the reaction of ethidium with the 30-kbp DNA could only be studied in the range of time constants $\tau > 70$ ms.

The reaction of DNA with ethidium in the stopped-flow instrument is reflected by a particularly large increase in the fluorescence, because in this case the degree of binding increases from zero to the equilibrium value. Due to the large reaction amplitude, the accuracy of the stopped-flow data is clearly higher than that of the data from T-jump experiments, where the extent of the reaction remains much smaller. While the main relaxation effects observed in our temperature-jump experiments could be described by single exponentials reasonably well, the corresponding stopped-flow reaction curves clearly require two exponentials for satisfactory fits (cf. Figure 6). The two time constants differ by factors in the range of 2–3. Fits by two exponentials are required both for natural DNA and for the synthetic poly[d(A–T)]; thus, the complexity of the reaction cannot be assigned to base-pair heterogeneity.

Table I: Parameters for the Binding of Ethidium to Double-Helical DNA[a]

					T-jump		stopped flow	
nucleic acids	T (°C)	$K_{app}/10^5$ (M^{-1})	FF	P	$k^+/10^6$ (M^{-1} s^{-1})	$K/10^5$ (M^{-1})	$k^+/10^6$ (M^{-1} s^{-1})	$K/10^5$ (M^{-1})
DNA 500 bp	2						0.83 ± 0.08	5.2 ± 2.5
DNA 30000 bp	2						0.62 ± 0.12	3.3 ± 1.4
DNA 200 bp	12				2.65 ± 0.13	3.9 ± 1.2		
DNA 500 bp	12	4.2 ± 0.2	33.4 ± 1.0	2.27 ± 0.3	2.70 ± 0.13	5.7 ± 2.4	2.3 ± 0.2	4.6 ± 2.0
DNA 4228 bp	12				2.91 ± 0.15	3.4 ± 1.0		
DNA 30000 bp	12				2.67 ± 0.13	9.9 ± 7.3	2.0 ± 0.2	4.0 ± 2.0
poly[d(A–T)]	12				6.88 ± 0.34	21 ± 13	4.9 ± 0.4	4.8 ± 2.0
DNA 200 bp	24				6.42 ± 0.32	3.8 ± 0.9		
DNA 500 bp	24	2.6 ± 0.1	18.4 ± 1.0	2.5 ± 0.3	6.44 ± 0.3	3.9 ± 0.9	5.4 ± 0.5	1.4 ± 0.4
poly[d(A–T)]	24	4.3 ± 0.2	23.5 ± 1.0	1.7 ± 0.3	12.3 ± 0.6	17 ± 10	12.5 ± 1.3	3.7 ± 1.2

[a] The following parameters are from fluorescence titrations, evaluated according to the excluded site binding model: K_{app}, equilibrium constant; FF, fluorescence intensity of the bound relative to the free state of ethidium measured with an EMI 9635QA photomultiplier; P, number of base pairs occupied by ethidium. The rate constant of association, k^+, and the equilibrium constant, $K = k^+/k^-$, are derived from relaxation time constants according to eq 2.

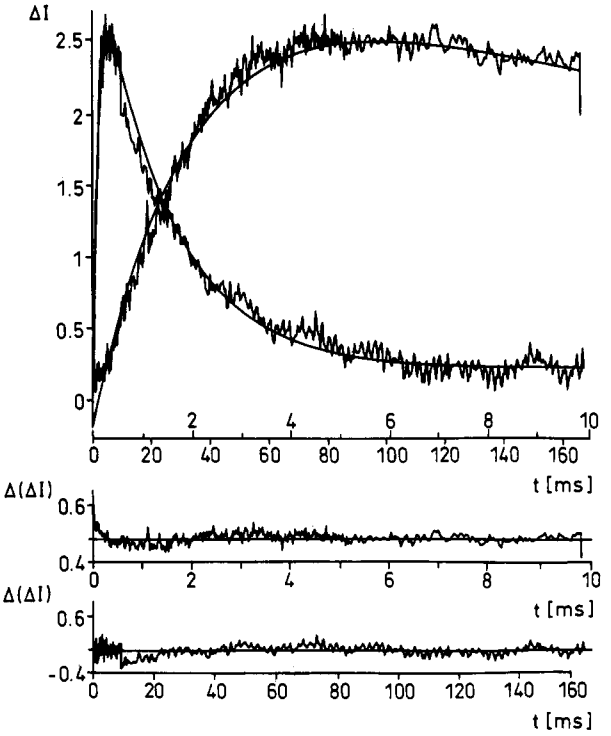

FIGURE 5: Temperature-jump relaxation observed at 50 μM poly[d(A–T)] and 3.33 μM ethidium at 12 °C shown at two different time scales (average of 18 individual jumps). The continuous line represents a least-squares fit with the time constants 2 and 25 ms. The two lower panels show the residuals of the two-exponential fit at the two time scales.

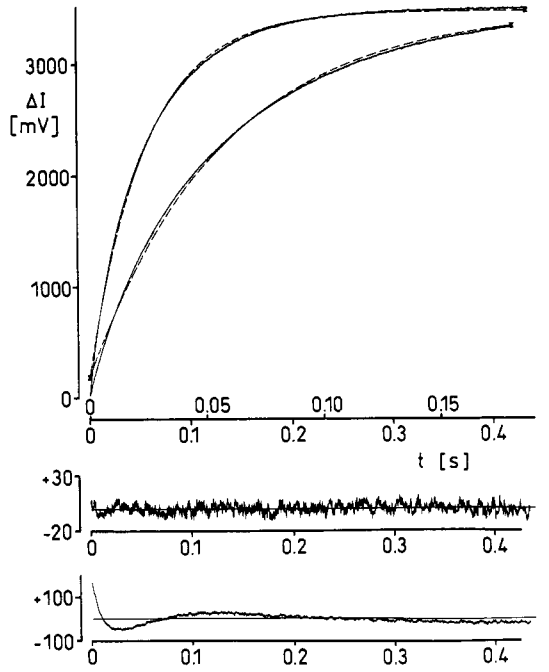

FIGURE 6: Stopped-flow experiment with 1.5 μM poly[d(A–T)] and 0.1 μM ethidium at two different time scales (12 °C). The top panel shows the experimental data (with some noise), a fit with two exponentials (indistinguishable from the experimental curve, τ_1 = 14.6 ms, τ_2 = 63.3 ms), and a fit with a single exponential (dashed line, τ = 57.2 ms). The two lower panels show the residuals of the two- and the one-exponential fits.

The deviation from the simple single exponential function also cannot be assigned to a second-order reaction, because the reaction is pseudo-first-order due to the large excess of DNA.

In spite of the clearly higher quality of the two-exponential fits, we have also used one-exponential fits of stopped-flow reaction curves in order to enable a direct comparison of the results obtained by the two methods. In all cases, the time constants obtained from one-exponential fits of T-jump and stopped-flow data were equivalent within the limit of experimental accuracy. This comparison shows that there is no special perturbation or any peculiar effect introduced by one of the methods. The reciprocals of both time constants obtained from the stopped-flow measurements show a linear increase with the DNA concentration (cf. Figure 7). The slopes derived from the data taken at 2 °C are 4.4×10^5 M^{-1} s^{-1} for the first and 1.2×10^6 M^{-1} s^{-1} for the second time constant. The corresponding intercepts are 0.1 and 2 s^{-1}, respectively; in general, the intercepts are less accurate than the slopes. Measurements on DNA samples with 500 bp and with 30 kbp do not reveal any chain length dependence of the two time constants within the limit of experimental accuracy. Finally, the time constants from two-exponential fits of T-jump relaxation curves—wherever available with some (limited) confidence—are consistent with corresponding data from the stopped-flow measurements.

Strong Dependence of Fluorescence Amplitudes on the Type of Photomultiplier. During our stopped-flow experiments it was found that the fluorescence amplitudes were much lower than expected according to the relative fluorescence changes observed in equilibrium titrations. This result seemed to indicate that a considerable part of the reaction occurred during the dead time of our stopped-flow instrument and suggested the existence of a separate fast part of the binding reaction. However, controls demonstrated that the reduced amplitude

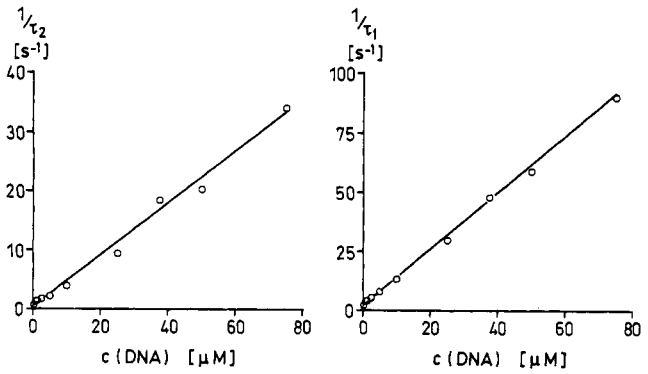

FIGURE 7: Results from two-exponential fits of stopped-flow data for the reaction of 500-bp DNA with ethidium at 2 °C fit to the cyclic reaction mechanism. The lines represent a fit by a simulation program; the rate constants are compiled in Table II.

was simply due to different types of multipliers used in the stopped-flow instrument (EMI 9558QB with a S20-photocathode sensitive up to 850 nm) and in the spectrofluorimeter (EMI 9635QA with a bialkali photocathode) used for the equilibrium titrations. Additional titration experiments showed that the fluorescence intensity of the DNA–ethidium complex relative to that of free ethidium is 12 with the EMI 9558QB and 18.4 with the EMI 9635QA (both values at 24 °C). The difference results from a steeper decay of the sensitivity in the long-wavelength range for the 9635QA than for the 9558QB. This difference affects the results because the fluorescence emission of ethidium is shifted to shorter wavelengths upon binding to DNA. The large variation of fluorescence enhancements reported in the literature for the ethidium–DNA system appears to be at least partly due to this effect.

3-Step Cyclic Reaction Scheme Consistent with the Experimental Data. Our measurements show that the reaction of ethidium with double-helical DNA is characterized by at least three relaxation processes. One of them has been observed only at high concentrations by the temperature-jump method and has not been detected by the stopped-flow technique. This process is associated with an amplitude opposite to that of the main binding reaction and appears to be due to some temperature-dependent conformational change. Because of its rather small amplitude we do not have information about its concentration dependence over a sufficiently wide range, and thus we have not attempted to model this process in detail.

The other two processes are more clearly identified by the stopped-flow measurements, but their existence is also indicated by the T-jump data. The relaxation time constants have been observed over a relatively wide range of concentrations, at various temperatures, and for different DNA samples including the synthetic poly[d(A–T)]: in all cases, the $1/\tau$ values show a linear increase with the reactant concentration ($\bar{c}_b + \bar{c}_e$) up to high $\bar{c}_b + \bar{c}_e$ values. This dependence is remarkable because it is not consistent with some simple mechanisms, which would appear to be appropriate for the present system. For example, standard reaction mechanisms such as

$$\text{DNA} + \text{ethidium} \underset{k_{\bar{1}}}{\overset{k_1^{\dagger}}{\rightleftharpoons}} C_1 \underset{k_{\bar{2}}}{\overset{k_2^{\ddagger}}{\rightleftharpoons}} C_2 \qquad (3)$$

or

$$\text{DNA} + \text{ethidium} \underset{k_{\bar{1}}}{\overset{k_1^{\dagger}}{\rightleftharpoons}} C_1 \qquad (4a)$$

$$\text{DNA} + \text{ethidium} \underset{k_{\bar{2}}}{\overset{k_2^{\ddagger}}{\rightleftharpoons}} C_2 \qquad (4b)$$

predict two processes, but one of the $1/\tau$ values goes to a constant level within the investigated range of concentrations, where amplitudes induced by temperature jumps are sufficiently high.

The results can be explained by a special cyclic reaction scheme which was proposed some time ago by Bresloff and Crothers (1975). Because the arguments in favor of this mechanism have been discussed already, the present discussion is restricted to the essential arguments. For the description of two relaxation processes we need two different binding states, C_1 and C_2. Bresloff and Crothers (1975) demonstrated that both $1/\tau$ values show a linear increase with the reactant concentration ($\bar{c}_b + \bar{c}_e$) when there is a direct coupling between the complex states, according to the following cyclic reaction scheme:

$$\text{DNA} + \text{ethidium} \underset{k_{\bar{1}}}{\overset{k_1^{\dagger}}{\rightleftharpoons}} C_1 \qquad (5a)$$

$$\text{DNA} + \text{ethidium} \underset{k_{\bar{2}}}{\overset{k_2^{\ddagger}}{\rightleftharpoons}} C_2 \qquad (5b)$$

$$C_1 + \text{DNA} \underset{k_{\bar{1}}}{\overset{k_1^{\dagger}}{\rightleftharpoons}} C_2 + \text{DNA} \qquad (5c)$$

The third reaction of this scheme is a conversion of the two complexes catalyzed by DNA, leading to a relatively fast coupling between the complexes. In the absence of the third step, C_1 and C_2 can be converted into each other only via dissociation of ethidium, which is a relatively slow reaction. The activation barrier is reduced by a direct transfer of ethidium ligands from occupied to free sites. In our calculations, the transfer reaction has always been modeled as an intermolecular step; intramolecular transfer is also possible, provided that the DNA molecules are sufficiently long.

We have used numerical procedures for the simulation of the experimental time constants. An example of a fit is given in Figure 7; the resulting rate constants are compiled in Table II. We do not show fluorescence amplitudes, because most of the amplitudes obtained by stopped-flow experiments are reduced due to the limited time resolution of the instrument. We have checked amplitudes resulting from temperature-jump relaxation curves which have been fit by two exponentials; these amplitudes are consistent with the 3-step cyclic mechanism and can be represented by relative fluorescence enhancements, which are very similar for the two ethidium–DNA complexes. Thus, our results are not in contrast with literature reports on fluorescence decays of ethidium–DNA complexes with single exponentials (Genest & Wahl, 1978; Millar et al., 1980).

DISCUSSION

The intercalation of aromatic residues into DNA double helices appears to be a relatively simple reaction, but as usual, the simplicity or complexity depends on the point of view of the observer and the methods used for observation. When a method is used with a relatively low accuracy, it is more likely

Table II: Parameters for the Binding of Ethidium to Double-Helical DNA According to the Cyclic Reaction Mechanism[a]

T (°C)	$k_1^+/10^5$ (M^{-1} s^{-1})	k_1^- (s^{-1})	$k_2^+/10^5$ (M^{-1} s^{-1})	k_2^- (s^{-1})	$k_t^+/10^5$ (s^{-1})	$k_t^-/10^5$ (s^{-1})
KT-DNA						
2	0.3	0.5	11.7	2	3.94	0.45
12	4.1	3	28.4	10	7.95	3.8
24	10.5	14	72.5	39	14.9	6
E_a (kJ mol^{-1})	109 ± 33	102 ± 8	56 ± 1	92 ± 8	41 ± 3	79 ± 32
poly[d(A–T)]						
12	10.5	13	80	50	21.8	11
24	30	25	400	70	85.7	18

[a] See text. The activation enthalpies, E_a, have been derived by Arrhenius plots.

that the results are consistent with a simple interpretation; conversely, at higher accuracies more details are usually detected, and more complex interpretations have to be given. In the case of the intercalation of ethidium into DNA, the stopped-flow data showed the highest signal to noise ratio; thus, two time constants associated with the increase of the fluorescence intensity upon ethidium binding to DNA could be characterized at a particularly high accuracy. In spite of the lower amplitudes resulting from T-jump experiments, the amount of information obtained from these experiments is relatively high: indications for the existence of more than a single relaxation process within the main fluorescence amplitude are apparent; moreover, another relaxation process with an amplitude of opposite sign has been identified. The results obtained by the two methods are consistent, and thus, it cannot be argued that these results are artifacts. As discussed above, the artifacts, which may be associated with Joule T-jump data and which have been accentuated by Marcandalli et al. (1984), were under rigorous control in the present investigation. The simple reaction progress curves presented by Macgregor et al. (1987), which could be fit by single exponentials, seem to be either due to a limited accuracy of the pressure-jump instrument used by these authors or due to selective perturbation of one binding mode by the pressure jump. The differences between our present observations and those of Macgregor et al. cannot be attributed to differences in the solvent conditions, because pH and ionic strength were virtually identical in these investigations.

Another controversial issue is the nature of the main intercalation step. Macgregor et al. (1987) concluded from measurements in solvents adjusted to different viscosities that the intercalation reaction is "controlled by a diffusive process". This conclusion is not consistent with our results on the chain length dependence. Our approach does not require any variation of the solvent; thus, it is less likely that our results are subject to artifacts. Macgregor et al. argue that their variation of the solvent does not involve any variation of important parameters other than the viscosity. However, it is clear that the presence of considerable amounts of methanol or glycerol in aqueous solutions may change essential parameters of a complex biopolymer like the DNA double helix, for example, via some change of the dielectric constant. It should be added that according to Macgregor et al. the contrast between the low rate constants and their control by a diffusive process is resolved by a model with a low fraction of about 10^{-3} of diffusive encounters leading to intercalation; the factor of $\sim 10^{-3}$ is assumed to represent the fraction of base pairs which are enabled by some conformational change to accept the intercalator.

Our results confirm the essential part of the data presented by Bresloff and Crothers (1975), which was later reanalyzed by Ryan and Crothers (1984). Similar conclusions were published by Wakelin and Waring (1980) for the binding of several phenanthridines and acridines to DNA. These authors did not report whether attention was given to potential artifacts resulting from field-induced orientation effects. Apparently, artifacts due to field-induced orientation were avoided by a particularly high salt concentration (1 M Na^+) used by Bresloff and Crothers (1975) as well as by Wakelin and Waring (1980). Test experiments with our 500-bp sample performed in our T-jump instrument with absorbance detection did not reveal evidence of orientation effects at 1 M Na^+. It should be mentioned, however, that our relaxation curves detected by fluorescence in our standard buffer did not show the "fast" relaxation process reported by Bresloff and Crothers with time constants around 50 μs. In spite of this difference, the existence of more than a single relaxation process and the linear dependence of the reciprocal relaxation times can be regarded as well documented. The most simple interpretation of these experimental data is the cyclic mechanism proposed by Bresloff and Crothers (1975), involving two different ethidium–DNA complexes and a direct transfer of ethidium between binding sites. The relatively fast transfer between the two different types of complexes appears to be in contrast with the NMR measurements of Feigon et al. (1982), who estimated an average lifetime of ~3 ms for ethidium–DNA complexes "somewhere between 26–40°C". Ryan and Crothers (1984) suggested a modified cyclic mechanism with binding of ethidium to two different sites on the DNA double helix, in order to resolve the apparent conflict.

At a first glance the cyclic mechanism with the direct transfer of the ligand between double helices appears to be rather complex. However, DNA double helices are complex structures, and the mechanism of intercalation is expected to be complex as well. In fact, the cyclic mechanism is already simplified, because none of the reaction steps can be regarded as simple elementary reactions. Intercalation is expected to be at least a 2-step reaction with formation of an outer complex in the first step and insertion of the ligand in the second step, as described by eq 3. Our experiments did not show direct evidence for *this* separation into two steps, which can be explained if the outer complex is relatively weak and is formed at a diffusion-controlled rate. The preequilibrium complex may be stabilized both by electrostatic and by some stacking interactions, which may lead to a preequilibrium constant of about 100 M^{-1}. The diffusion-controlled rate constant for binding of ethidium to DNA is expected to be in the range around 10^9 M s^{-1}. When we use these estimated values, the relaxation time constant for preequilibration is smaller than 1 μs, which is consistent with our observations (cf. the Temperature-Jump Experiments section).

For the case of fast equilibration of the outer complex, the observed rate constant of complex formation k_{app}^+ corresponds

to

$$k^{+}_{app} = K_p k_{int} \quad (9)$$

where K_p is the preequilibrium constant and k_{int} is the rate constant for the intercalation reaction. When K_p is again assumed to be 100 M^{-1}, the k_{int} value for the first type of complexes formed with natural DNA is about 300 s^{-1} at 2 °C and about 1×10^4 s^{-1} at 24 °C; using the same K_p value, the intercalation rate constants for the second type of complexes are approximately 1×10^4 s^{-1} at 2 °C and 7×10^4 s^{-1} at 24 °C. It is possible that the intercalation rate is determined by the rate of unstacking of base pairs, but it cannot be excluded that the activation barrier of unstacking is reduced by the approach of the ligands. These details of the intercalation mechanism could be analyzed, if preequilibrium constants were available and if corresponding data could be determined for various aromatic residues. Ramstein et al. (1980) have reported a 2-step mechanism for the intercalation of proflavin into poly[d(A–T)] double helices; their intercalation rate constant of 3.6×10^3 s^{-1} (at 17 °C) is equivalent to our value measured at 12 °C for the first type of ethidium complex, when we assume a preequilibrium constant K_p = 290 M^{-1}. Thus, the results seem to be consistent.

A problem raised by our results appears to be the nature of the two types of intercalation complexes. Because two types of complexes are observed also for poly[d(A–T)], the explanation cannot be based on a heterogeneity of binding sites on the double helix. However, we may expect two types of complexes resulting from two different orientations of the ethidium ligand in the DNA double helix. According to the X-ray structure of Tsai et al. (1977), the phenanthridinium residue is inserted between the base pairs, and the phenyl residue is located in the narrow groove of the double helix. In solution, ethidium molecules may also be inserted into the opposite direction with the phenyl residue in the wide groove. In fact, the ethidium residues stacked between the small double helices consisting of two base pairs, which have been used by Tsai et al., are arranged in this opposite direction. The two orientations are not equivalent, because the contacts between the aromatic residues are different and, thus, the stacking energy may also be different. In the case of ethidium, a difference may also result from different contacts of the phenyl residue in the minor and major grooves. This interpretation is equivalent to that presented by Wakelin and Waring (1980).

Finally, the apparent contrast between the NMR lifetime reported by Feigon et al. (1982) and the high transfer rate constants should be explained. It is very likely that the transfer reaction is a 2-step reaction in both directions with fast formation of a preequilibrium complex in the first step followed by a slow transfer of the ligand between the sites in the second step. Moreover, it is likely that the formation of the preequilibrium complexes does not induce large changes in the NMR spectrum. Because the transfer rate derived from the T-jump experiments is the product of the preequilibrium constant K_p and the transfer rate constant (see above), a K_p value of about 100 M^{-1} is sufficient to obtain an intrinsic transfer rate constant in the range of 10^3 s^{-1}, which is consistent with the NMR data. Thus, the modification of the cyclic mechanism proposed by Ryan and Crothers (1984) is not necessary for explanation of the NMR results.

In summary, all of the relevant data appear to be consistent. Experimental data obtained by the Joule temperature-jump technique are not perturbed by artifacts, provided that appropriate care is taken during the measurements. The "simple" results obtained by some techniques appear to be either due to selective perturbation of one of the complexes or simply due to a limited sensitivity. The complex reaction mechanism found for the intercalation of ethidium into DNA double helices represents no more than the reaction steps to be expected in this relatively complex system.

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SUPPLEMENTARY MATERIAL AVAILABLE

Theoretical basis of the measurements under magic angle conditions (8 pages). Ordering information is given on any current masthead page.

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