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SUBSTITUENT EFFECTS ON THE BINDING OF ETHIDIUM AND ITS DERIVATIVES TO NATURAL DNA

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The binding of eight ethidium derivatives to short (~ 35 base-pair), random sequence DNA has been investigated using ^{1}H-NMR. At 35°C, all drugs cause upfield shifts of the DNA imino proton resonances characteristic of intercalative binding to DNA, but the line shapes vary significantly with the nature of the drug. The results confirm our previous proposal that removal of the amino group at position-3, but not at position-8, on the parent ethidium shortens the lifetime of the intercalative state (less than 1–2 ms at 35°C). These results suggest that hydrogen-bonding interactions with the 3-NH_2 group are involved in stabilization of the drug-DNA complex or that changes in charge distribution that accompany removal of the 3-NH_2 group reduce the complex stability. The magnitude of the shift of the drug-DNA spectra indicates a slight preference for binding of the drugs adjacent to G·C base-pairs.

1. Introduction

In previous papers we used ^{1}H-NMR to investigate various aspects of the binding of a number of drugs to DNA [1,2]. More recently we used these techniques to investigate the interaction of a series of bisacridines to a synthetic DNA, $d(AT)_5$ [3,4]. These studies and work by other groups [5–11] have demonstrated that NMR measurements may be used to examine various aspects of drug-DNA interactions including mode of binding, sequence specificity, and kinetics of drug binding. In the present study we use NMR to examine the binding of a series of ethidium derivatives to approx. 35 base-pair DNA fragments. Ethidium was chosen for this study because it is one of the more widely studied drugs [12–14] and has played an important role as a model for drugs that bind to DNA by intercalation. In a preliminary NMR study of ethidium and two derivatives, we found that the removal of the 8-amino group had little effect on the ethidium-binding properties, but removal of the 3-amino group greatly affected the lifetime of the drug in any particular intercalation site [1]. In the present study, we have extended these measurements to a wider range of ethidium derivatives to test the effect of substitutions at these and other positions on the DNA-binding properties of ethidium.

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2. Experimental

The preparation of short, random sequence DNA from chicken erythrocyte chromatin is described elsewhere [2]. For the NMR experiments the DNA was dialyzed vs. 10 mM sodium cacody-

late, pH 7.0, 0.1 M NaCl, 10 mM $MgCl_2$. This buffer system was chosen, since it is believed to disfavor outside binding relative to the intercalative mode of binding [15]. The size distribution of the DNA was determined by polyacrylamide gel electrophoresis and by ^{31}P-NMR (ratio of terminal to interior phosphates). At least 90% of the DNA is between 15 and 100 base-pairs long, with a median molecular length (weight average) of approx. 35 base-pairs. Ethidium bromide was obtained from Sigma Chemical Co. and 3 was a gift from Professor H.W. Zimmermann. The synthesis of the other compounds will be described elsewhere.

Samples were contained in Wilmad 508 cp microcells. 120 μl of approx. 40 mM base-pair DNA was pipetted into the tube using a pulled Pasteur pipet, which was reused to withdraw samples for drug addition. Drugs were dissolved in ethanol and concentrations were determined by visible absorption spectroscopy [2], and then appropriate amounts were pipetted into small Eppendorf centrifuge tubes and dried under a stream of N_2 gas. To add drugs, the DNA solution was removed from the NMR tube, added to the Eppendorf tubes, mixed until the drug was dissolved, and then returned to the NMR tube. With the high DNA concentrations used in these experiments, all added drugs are completely bound to the DNA [16,17].

Spectra were obtained using a Varian HR 300 proton spectrometer, modified to operate as a correlation spectrometer. With approx. 3 mg of DNA in 120 μl, a typical low-field spectrum required 23 min of signal averaging. Resonance positions are in ppm downfield relative to the standard TSP (sodium 3-trimethylsilylpropionate).

3. Results and discussion

The structures of the eight ethidium derivatives investigated are shown in fig. 1 and table 1. Figs. 2 and 3 show the effects of binding of these drugs on the low-field spectra of the DNA at 35°C. The two partially resolved envelopes in the low-field spectra of the free DNA (no drug) are due to a collection of overlapping resonances (> 30 Hz linewidth) from individual hydrogen-bonded G and T imino protons in G · C (higher-field peak) and A · T (lower-field peak) base-pairs, respectively [18]. When a drug intercalates into the DNA, the ring current shift originally exerted on the base-pairs at the intercalation site by one of the neighboring base-pairs is replaced by the generally larger ring current shift from the drug [19,20]. We therefore expect resonances from base-pairs at the intercalation site to be shifted upfield and for a large number of intercalating drugs that we have studied this is the general result [2]. Downfield

Table 1

The DNA-binding parameters of ethidium derivatives

Compound no.	Substituents				K [a] (M^{-1})	Unwinding [b] angle (°)	Kinetics at 35°C
	R_3	R_8	R_6	R_5			
1	NH_2	NH_2	C_6H_5	C_2H_5	5×10^5	26	slow
2	NH_2	NH_2	C_6H_5	CH_3	5×10^5	26	slow
3	NH_2	H	C_6H_5	C_2H_5			slow
4	NH_2	NH_2	CH_3	C_2H_5	5×10^5	26	slow (intermediate)
5	H	NH_2	C_6H_5	C_2H_5	4×10^5		fast
6	H	NH_2	C_6H_5	CH_3			fast
7	H	NH_2	p-NH_2-C_6H_4	C_2H_5	5×10^5	23	fast
8	$N(CH_3)_2$	$N(CH_3)_2$	C_6H_5	C_2H_5	1×10^4		c

[a] Ref. 31.
[b] Ref. 32.
[c] Little or no binding evident in NMR.

shifts are observed for nonintercalative binding of drugs in the minor groove [2]. The results in figs. 2 and 3 show that with the possible exception of **8** all drugs cause upfield shifts, but there are large differences in the magnitudes of the shifts and in the overall character of the resulting spectra. For example, with the addition of increasing levels of compounds **1**, **2**, **3**, and perhaps **4**, the A · T and G · C resonances shift slightly upfield and appear to broaden considerably. Quite different behavior is observed with des-3-amino derivatives (**5**, **6**, **7**), which produce large upfield shifts, and considerably less broadening of the spectra even at high drug levels.

Since these compounds all have the same chromophore and bind to DNA by intercalation, their different behavior might seem surprising. However, a study of the temperature dependence of the low-field spectra of drug-DNA complexes with **1**, **3** and **5** has shown that most of the differences noted at 35°C are due to differences in the lifetimes of the three derivatives in the intercalated state, rather than to variations in the mode of binding or geometry in the complex. When the lifetime of the intercalated drug in a particular binding site, τ, is shorter than 1 ms (fast exchange), all resonances in the DNA spectra are upfield shifted to a degree that is proportional to the fraction of time a drug occupies a neighboring site [1]. Consequently, there is a continuous shift of the entire spectrum with increasing amount of drug. This behavior is observed for compounds **5**, **6** and **7**. When the drug is in the intermediate exchange regime, $\tau = 2$–3 ms, there is uncertainty-principle broadening of the drug-shifted resonances and for ethidium, **1**, and compounds **2**, **3** and **4** this occurs around 35°C. Finally, in the slow-exchange regime where $\tau \gtrsim 10$ ms, separate upfield-shifted resonances can be observed for the drug-DNA complex and in the case of ethidium, **1**, this condition was obtained at 17°C [1]. We therefore conclude that the very different spectral behavior exhibited by compounds **1**, **2**, **3** (and perhaps **4**) as compared with compounds **5**, **6** and **7** can be attributed to differences in binding site lifetimes. This supports our previous suggestion that it is the NH_2 group at the 3-position rather than at the 8-position that is crucial to increasing the lifetime of the drug binding site lifetimes [1].

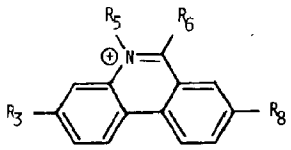

Fig. 1. Structures of the ethidium derivatives examined.

For those drug-DNA complexes that are in fast exchange the upfield shifts are larger for the G · C resonances than for the A · T resonances as shown graphically in fig. 4 [1,2]. Assuming that A · T and G · C resonances are shifted by about the same amount when ethidium binds at an adjacent site, we would conclude that there is preference for binding of compounds **5**, **6** and **7** adjacent to G · C base-pairs. For compound **5** the preference is small, but for compound **6** there might be as much as a 2-fold preference for G · C over A · T. Our previous studies gave similar results for the ethidium-DNA complex at elevated temperatures [1]. At 58°C ethidium is also in fast exchange and the G · C resonances of DNA with 1 ethidium per 6.2 base-pairs are shifted further upfield than the A · T resonances (relative to the free DNA spectra) by a factor of about $(0.57/0.4) = 1.4$. Compound **7** exhibited behavior that is intermediate between **5** and **6**.

Compound **8**, in which both amino groups have been substituted with methyl groups, has little effect on the low-field spectrum of DNA except at the higher levels. In an earlier study of the binding of ethidium derivatives, Jacquemin-Sablon et al. [21] found that the DNA binding constant for this compound is about 50-times smaller than for ethidium and by other criteria does not appear to intercalate.

3.1. Relationship to previous studies

Early studies of the kinetics of ethidium binding by temperature-jump [22,23] and stopped-flow techniques [24] indicated that the rate constant for dissociation of ethidium from DNA is of the order of 50–200 s^{-1}, and this is to be compared with our results which indicate that the off-rate for ethidium as well as **2**, **3** and **4** is of the order of 100–300 s^{-1}

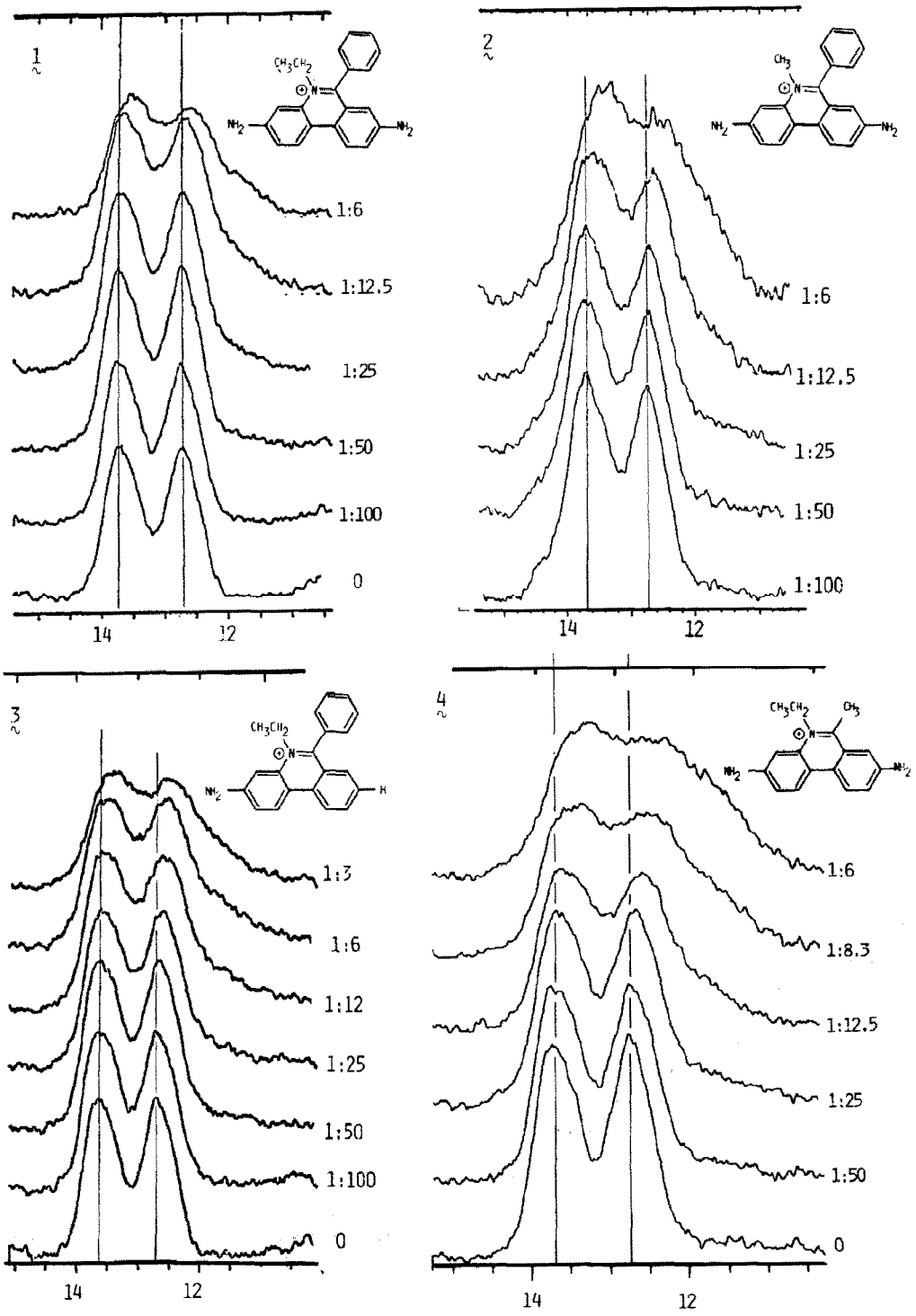

Fig. 2. ^{1}H-NMR spectra of the low-field resonances of DNA as a function of added drug at 35°C. The drug per base-pair ratio is indicated at the right of each spectrum. These ethidium derivatives all contain an NH_2 group at the 3-position and are in slow-to-intermediate exchange.

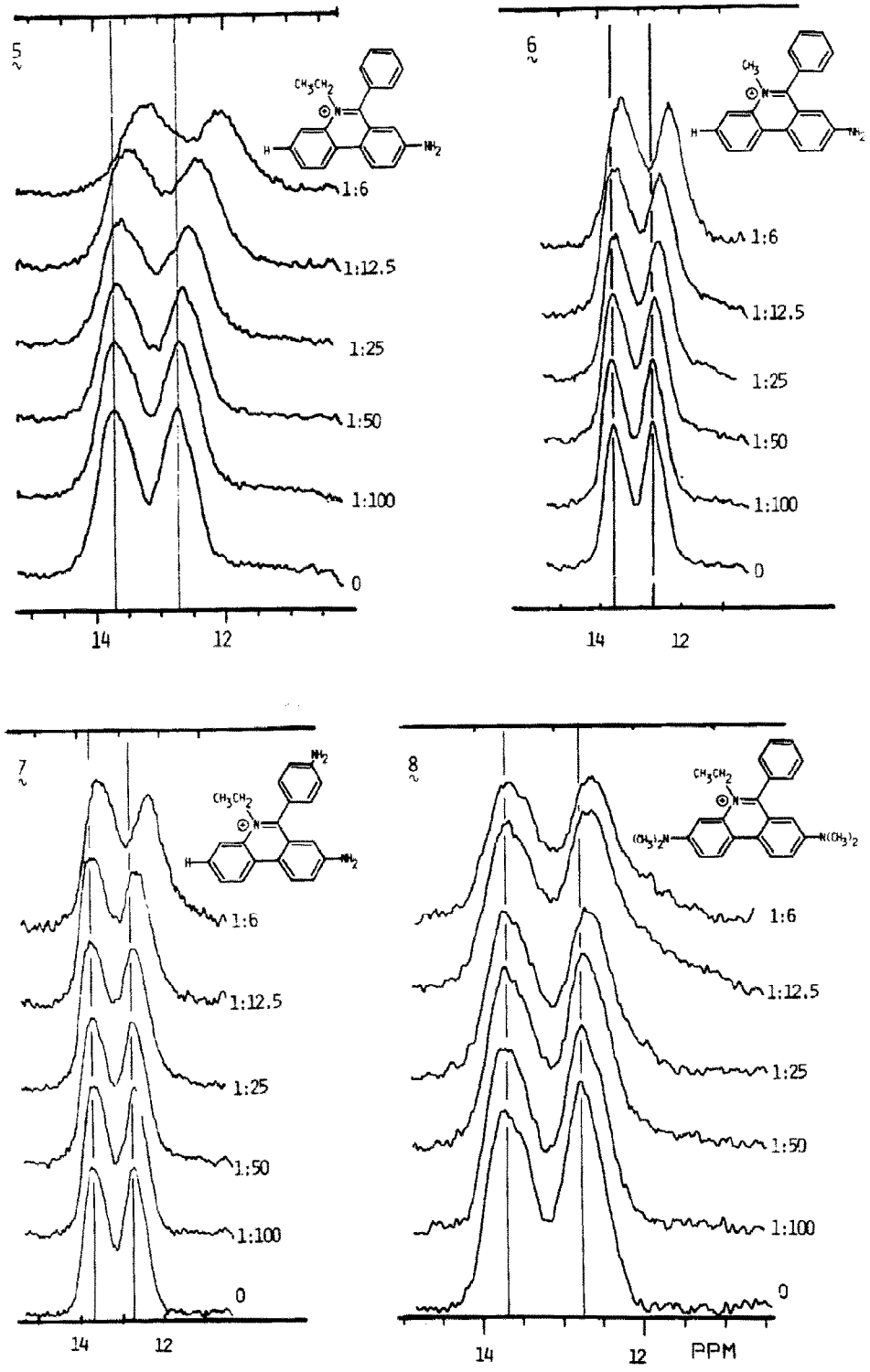

Fig. 3. ^{1}H-NMR spectra of the low-field resonances of DNA as a function of added intercalating drug at 35°C. These ethidium derivatives lack the NH_2 group at the 3-position and are in fast exchange.

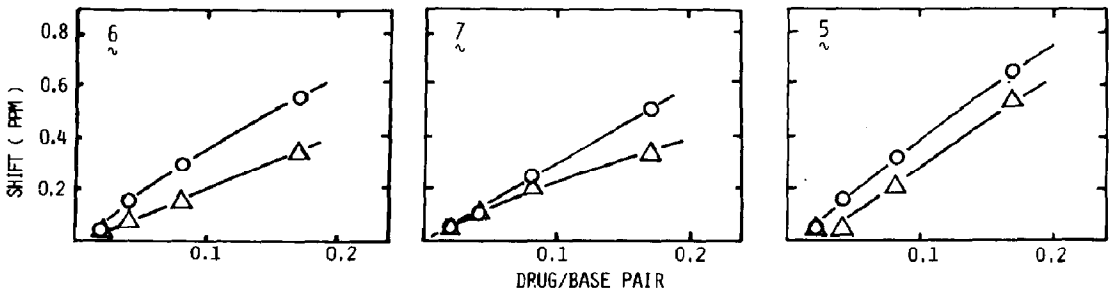

Fig. 4. Plots of the average chemical shifts of the A·T (△) and the G·C (○) resonances for those intercalators that exhibit fast exchange kinetics at 35°C.

at 35°C. In recent studies, Chandrasekaran et al. (unpublished results) directly compared stopped-flow and NMR measurements of the kinetics of ethidium binding to poly(dG-dC) and found very good agreement between the off-rates measured by the two techniques. When first reported, our observations on the ethidium-binding kinetics appeared to be a disagreement with the earlier temperature-jump experiments of Bresloff and Crothers [23]. They reported a bimolecular rate constant of 2×10^6 M^{-1} s^{-1} for the intermolecular transfer of ethidium between DNA molecules. In our solutions, which were approx. 40 mM in base-pairs, this would have resulted in a binding site lifetime of only 10^{-5} s, a value which is totally incompatible with our NMR results. Subsequently, Ryan and Crothers [25] reanalyzed the temperature-jump measurements and found that using a different model they could fit their data using a much smaller value for the intermolecular transfer rate constant, thus removing the apparent discrepancy. Our observations regarding the binding of ethidium bromide to DNA and our interpretation of the kinetic aspects of the binding have been confirmed by the recent studies of Chandrasekaran et al. [26]. Several years ago, Hogan and Jardetzky [27] reported that there is a stoichiometric loss of intensity in the NMR spectrum accompanying addition of ethidium to DNA. Our studies, and those of Jones and Wilson [28], Wilson et al. [8] and Levy et al. [9] do not confirm this earlier report. We note that when concentrated solutions of ethidium are added to concentrated DNA solutions formation of aggregates may take place, and this could explain the observations of Hogan and Jardetzky [27].

Previous studies of the crystal structure of ethidium complexes with UpA and CpA [29,30] suggest that both amino groups of ethidium have weak hydrogen-bonding interactions with phosphate and ribose groups, implying that both amino groups might be important to the binding. Our NMR studies as well as previous studies by Wakelin and Waring [22] on the kinetics of binding ethidium and a des-3-aminoethidium derivative clearly identify the amino at the 3-position as the crucial one in stabilizing the complex. It will be interesting to see whether molecular mechanics calculations are consistent with these experimental observations regarding substituent effects on ethidium binding to DNA.

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