^{1}H NMR Study of the Binding of Bis(acridines) to d(AT)$_5$·d(AT)$_5$. 1. Mode of Binding†

Nuria Assa-Munt,‡ William A. Denny,§ Werner Leupin,‖ and David R. Kearns*
Department of Chemistry, University of California—San Diego, La Jolla, California 92093
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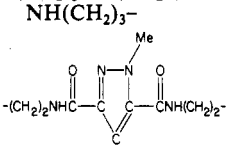
ABSTRACT: ^{1}H NMR has been used to investigate the mode of binding to d(AT)$_5$·d(AT)$_5$ of a series of bis(acridine) derivatives connected by different types of linker chains. The length and character (ionic, aliphatic, rigid, and flexible) of the linker chains are found to have a profound effect on the binding of these derivatives to the DNA. Bis(acridine) derivatives with linker chains shorter than 9 Å monointercalate under the conditions used in the NMR study, whereas those bis(acridines) with chains of 9.8 Å or longer bisintercalate. We find no evidence for the violation of the so-called neighbor exclusion principle. Although all of the bis(acridines) contain the same chromophores, their NMR spectra clearly demonstrate that they form complexes with d(AT)$_5$·d(AT)$_5$ which have different structures. This emphasizes the important effect that the linker chain has on the structure of the intercalation complex.

The chemical and biochemical properties of intercalating molecules such as actinomycin D, proflavin, ethidium bromide, and their derivatives have long been an area of active research (Albert, 1966; Wilson & Jones, 1982). These drugs intercalate into DNA helices (Lerman, 1961; Sobell, 1980; Berman & Young, 1981; Waring, 1981), and some (e.g. actinomycin D) exhibit antitumor activity. While binding to DNA is not the sole factor determining their chemotherapeutic properties, the antitumor effectiveness for several families of intercalating drugs is correlated with their DNA binding affinity (Le Pecq et al., 1974; Baguley et al., 1981; Denny et al., 1982a). In view of this, many laboratories (Le Pecq et al., 1975; Canellakis et al., 1976; Cain et al., 1978; Gaugain et al., 1978; Wakelin et al., 1978; Becker & Dervan, 1979; Kuhlmann & Mosher, 1981) have synthesized drugs containing two chromophores joined by a molecular linker chain. The most widely studied class of potential bisintercalating agents has been the bis(acridines) of general structure shown in Figure 1A and Table I. Compounds of this class have been prepared by several groups as potential antitumor agents (Fico et al., 1977; Chen et al., 1978; Denny et al., 1984a,b) and as probes of bifunctional ligand/DNA interaction (Lown et al., 1978; Capelle et al., 1979; Wright et al., 1980; King et al., 1982; Delbarre et al., 1983). A primary goal of the latter studies has been to determine the importance of both the length and the nature (flexibility, polarity, H donor or acceptor ability, etc.) of the linker chain on the binding parameter of a potential bisintercalator.

Le Pecq et al. (1975) have postulated that bisintercalative binding occurs only when the linker chain length is 10.2 Å or longer, allowing the chromophores to occlude (sandwich) two base pairs between them (see Figure 1, model III). In support of this, King et al. (1982) find that a highly rigid bis(acridine) with an 8.8-Å separation between chromophores monointercalates. However, evidence from viscometric studies using both the unwinding of closed circular supercoiled DNA and the helix extension of short linear DNA indicates that bis(acridine) (**2**) linked by a polymethylene chain of 8.8-Å length (Wakelin et al., 1978) and **6** linked by a monoamide chain of 8.6-Å length (Denny et al., 1984a,b) bind bisintercalatively. Under the assumption of Fuller–Waring type geometry, these ligands bind in violation of the "neighbor exclusion principle", with one chromophore positioned on either side of a single base pair (Crothers, 1968; Bauer & Vinograd, 1970) (see Figure 1, model II). Wakelin & Waring (1976) reported that echinomycin binds to poly[d(AT)] with a binding site size of three base pairs which precluded site exclusion and indicated binding in contiguous sites (Figure 1, model II). However, a subsequent theoretical analysis (Shafer & Waring, 1980) of these data demonstrated that in cases of mixed single and double

Table I: Structure and Nomenclature of Bis(acridine) Compounds

compd no.	nomenclature	atoms in linker chain	linker:[a] R =	length of R (Å)[a]
1	C_4	4	$-(CH_2)_4-$	6.3
2	C_6	6	$-(CH_2)_6-$	8.8
3	C_7	7	$-(CH_2)_7-$	10.0
4	C_8	8	$-(CH_2)_8-$	11.3
5	C_{10}	10	$-(CH_2)_{10}-$	13.8
6	Amd-6	6	$-(CH_2NHC(=O)(CH_2)_2-$	8.6
7	Amd-7a	7	$-(CH_2)_2NHC(=O)(CH_2)_3-$	9.8
8	Amd-7b	7	$-(CH_2)_3NHC(=O)(CH_2)_2-$	9.9
9	Amd-8	8	$-(CH_2)_3NHC(=O)(CH_2)_3-$	11.1
10	spermidine	8	$-(CH_2)_3NH(CH_2)_4-$	11.2
11	spermidine (BAS)	12	$-(CH_2)_3NH(CH_2)_4NH(CH_2)_3-$	16.1
12	pyrazole (BAPY)	11		13.7

[a] N to N distance.

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‡ Present address: The Salk Institute, La Jolla, CA 92138.
§ Permanent address: Cancer Chemotherapy Research Laboratory, University of Auckland School of Medicine, Private Bag, Auckland, New Zealand.
‖ Permanent address: Institut fur Molekularbiologie und Biophysik, ETH-Honggerberg, CH-8093 Zurich, Switzerland.

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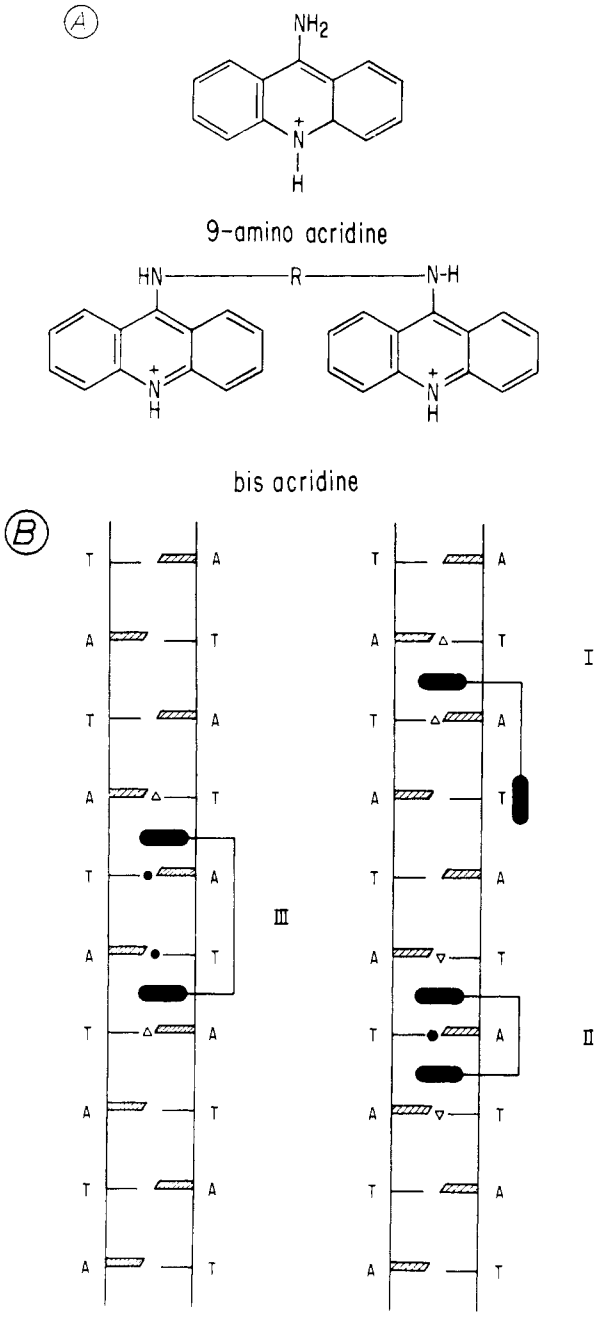

FIGURE 1: (A) Structure of 9-aminoacridine and the general bis(acridine) structure. (B) Models for the binding of bis(acridines) to $(AT)_5$. I, monointercalation; II, bisintercalation, contiguous occupation of binding sites; III, bisintercalation binding of chromophores with site exclusion. The solid symbols indicate interior protons, most shifted upfield by drug, whereas open triangles denote protons less shifted upfield by drug binding.

intercalation, a site size of less than four base pairs could be obtained, without it implying any violation of the neighbor exclusion principle. Delbarre et al. (1983) have presented ^1H NMR evidence for bisintercalation of bisethidium spermidine with poly(dA-dT), but insolubility of the complex necessitated use of high temperatures (>60–90 °C) and low drug per phosphate levels; this resulted in small (0.04 ppm) shifts of the DNA resonances. There was the additional complication that shifts of the sugar resonances were larger than those of the base resonances, making it difficult to distinguish between different modes of binding. At this point then, the only suggestion that the excluded site model is violated arises from studies by Wakelin et al. (1978) and Atwell et al. (1983). We note, however, the methods used to determine intercalation are indirect methods in which properties of new compounds are compared to "standard" established intercalators like ethidium bromide (e.g., changes in the sedimentation coefficients, viscosity, or mobility in the gels as a function of addition of drugs to sonicated, linear DNA or superhelical circular DNA). These measurements indicate the extension of the DNA helix and/or of the local unwinding of superhelical circular DNA caused by intercalation of the drug, but none of these tests by itself is a conclusive indicator of intercalation (Waring, 1981). For example, certain nonintercalative drugs (e.g. netropsin, irehdiamine A, and distamycin) can lengthen the DNA helix, change the sedimentation coefficient, and cause helix unwinding (Dougherty, 1982). Thus, lengthening and unwinding angle measurements alone cannot unequivocally determine if a drug intercalates. Moreover, the unwinding angles for "well-established" intercalators vary from 26° for ethidium bromide to 11° for daunomycin (Waring, 1981).

Flexibility of the linker chain also plays an important role in determining binding mode. Flexible chains permit considerable self-stacking of the acridine chromophores in solution (Gaugain et al., 1978), and this process has been suggested to hinder bisintercalative binding (Capelle et al., 1979). To decrease this self-association, bis(acridines) with less flexible linker chains have been prepared, and indeed, the pyrazole bis(acridine) (**12**) binds bisintercalatively (Denny et al., 1984b).

In the present study we use high-field ^1H NMR spectroscopy to study the binding of the bis(acridines) listed in Table I to the synthetic deoxyribonucleotide $d(AT)_5$·$d(AT)_5$. When the large (>1 ppm) perturbations of the low-field imino protein resonances which occur on ligand addition are monitored, details of the mode of binding and kinetics of the bound chromophores can be determined as we have demonstrated in previous studies of drug binding to short duplexes of natural DNA (Feigon et al., 1984). In particular, the method is able to effectively distinguish between mono- and bisintercalation of the ligands and, in appropriate cases, distinguish between possible modes of bisintercalation. We are, therefore, able to determine the minimum length of a linker chain connecting two chromophores necessary for bisintercalation to occur and test for bisintercalation which violates the so-called neighbor exclusion principle. The effect that the nature of the linker chain has on the way in which the two chromophores intercalate is also evident in the NMR spectra. A preliminary account of this work was first presented elsewhere (Assa-Munt et al., 1982), and in the following paper we discuss the dynamic aspects of the binding (Assa-Munt et al., 1985).

Experimental Procedures

Materials. The bis(acridines) of Table I were prepared in the Cancer Research Laboratory, Auckland, and their synthesis will be reported later (Denny et al., 1984a). All compounds were used as hydrochloride salts and were homogeneous on thin-layer chromatograms. Compounds **2–5**, **10**, and **11** (Chen et al., 1978) were crystallized as hydrochlorides from anhydrous solvents and assumed to be anhydrous forms. Compounds **6–9** and **12** were made up into solution by using the formula weights determined by elemental analysis (Denny et al., 1984a,b). $d(AT)_5$·$d(AT)_5$ was prepared by a modified phosphotriester synthesis in liquid phase (Denny et al., 1982b) as the sodium salt.

Methods. Unless otherwise specified, all NMR spectra were determined on a Varian HR 300-MHz proton spectrometer modified to operate as a correlation spectrometer. A total of 120 μL of a 2 mM (in duplex) solution of $d(AT)_5$·$d(AT)_5$ in 0.1 M NaCl and 0.01 M sodium cacodylate buffer at pH 7.0

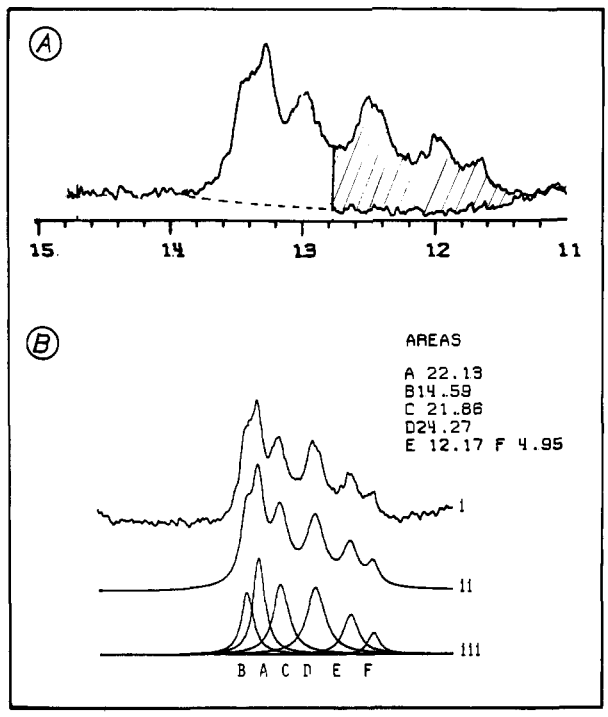

FIGURE 2: Illustration of two methods used to determine the relative intensity of upfield shifted resonances in the spectrum of a 1:1 complex of **12** with $d(AT)_5 \cdot d(AT)_5$. In (A), the total area of the spectrum was determined first by cutting and weighing the entire spectrum. The shaded portion of the spectrum was then cut out and separately weighed and compared with the total. This yielded a value of 40% shifted intensity. In (B), the observed spectrum (top) was simulated (middle) by superimposing a sum of resonances with positions and intensities shown at the bottom. The relative intensity of the peaks (D + E + F) corresponds to 41.4% of the total intensity.

was placed in a Wilmad microcell (120 μL) and the spectrum of the free oligonucleotide determined. For serial drug addition, bulk solutions of drug of known concentration were made up in water or aqueous ethanol, and the appropriate amounts to correspond to a cumulative ratio of 1:5, 1:2, and 1:1 drug molecules per base pair when dissolved in 120 μL were pipetted into 0.5-mL Eppendorf tubes. The solvents were evaporated in a stream of dry N_2. After the spectrum of the free oligonucleotide sample was recorded, it was removed from the microcell, placed in the first Eppendorf tube, and vortexed until all the drug had dissolved. The sample was returned to the microcell and equilibrated for 15 min, the spectrum was determined, and the process was repeated.

The titrations of $d(AT)_5 \cdot d(AT)_5$ with drugs were carried out at 6 °C since all the imino protons of the decamer duplex could be observed at this temperature. Because of the high binding constants of these compounds ($K > 10^8$ M^{-1}), all of the drug was bound to DNA under our experimental conditions, and at 0.1 M NaCl, intercalative binding was favored over outside binding modes (Jones et al., 1980).

Upon binding of drugs, imino proton resonances in the $d(AT)_5 \cdot d(AT)_5$ spectrum were shifted upfield. To determine the intensity of shifted resonances relative to the total intensity, two different integration techniques were used. In the first method, we simply cut out and weighed the appropriate regions of the spectra as illustrated schematically in Figure 2A. In the second method, the observed spectrum of the drug–DNA complex was simulated by using the Nicolet software, and the integrated intensities of the shifted peaks relative to the total integrated intensity were then determined by the Nicolet program. An example of this approach is illustrated in Figure 2B.

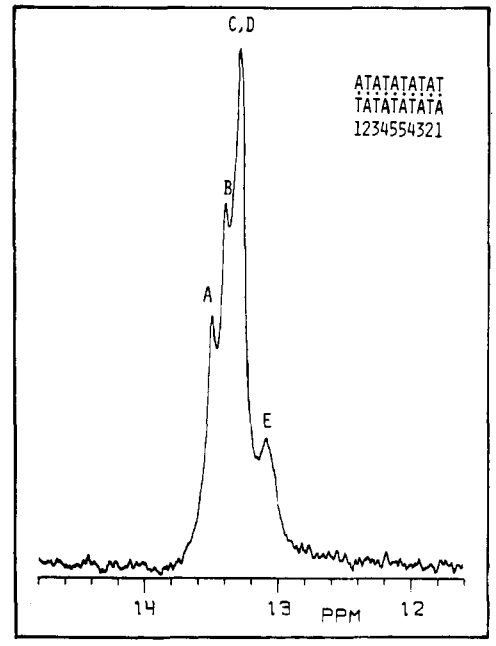

FIGURE 3: Low-field 300-MHz NMR spectrum of $d(AT)_5 \cdot d(AT)_5$ at 6 °C. The sequence of the decamer studied and numbering system used is indicated. The assignments are as follows: A (no. 2), B (no. 3), C, D (no. 4, 5), E (no. 1).

RESULTS

Free $d(AT)_5 \cdot d(AT)_5$. Figure 3 shows the low-field spectrum of the decadeoxyribonucleotide nonaphosphate $d(AT)_5 \cdot d(AT)_5$ at 6 °C. The presence of resonances due to the H-bonded imino protons of the T residues in aqueous solution demonstrates that the molecule is in the duplex form, where the protons are protected from exchange with the solvent (Kearns, 1977). At this temperature, it is evident that resonances from all 10 base pairs are observed. From the temperature dependence of the resonances (Assa-Munt, 1983; Assa-Munt et al., 1985), it can be concluded that the highest field resonance at 13.1 ppm belongs to the imino protons of the terminal base pairs, which have been upfield shifted by exchange due to "fraying" (Patel & Hilbers, 1975) even at this low temperature. Note that the exact position of this resonance is very sensitive to temperature and because of small variations in temperature the highest field resonance in the spectrum is sometimes "buried" under the main collection of resonances. The assignments of the other resonances are indicated in Figure 3 and discussed in more detail in the following paper (Assa-Munt et al., 1985).

9-Aminoacridine (9AA) Complexes. Figure 4 shows the low-field spectrum of the complex of $d(AT)_5 \cdot d(AT)_5$ with 9-aminoacridine at different ligand:duplex ratios. As drug is added to $d(AT)_5 \cdot d(AT)_5$, the new intensity appears at ~12.3 ppm, and there is a decrease in the intensity of resonances at ~13.2 ppm. At relatively high drug levels (1:2) even the original 13.2 ppm peak shifts slightly upfield. The origin of this shift is discussed in the following paper (Assa-Munt et al., 1985). As noted earlier (Feigon et al., 1982, 1984) the generation of upfield shifted peaks is indicative of intercalative binding, in which the moderate shielding effect of an adjacent base pair is replaced by the somewhat greater shielding of the ligand chromophore. The intensity of the shifted peak relative to the unshifted peak is an important diagnostic monitor of the drug binding properties. With one drug per duplex, 2 resonances out of a possible 10 (20%) will be shifted upfield for each chromophore bound (monointercalation). When *two* chromophores intercalate, 40% of the resonance intensity will

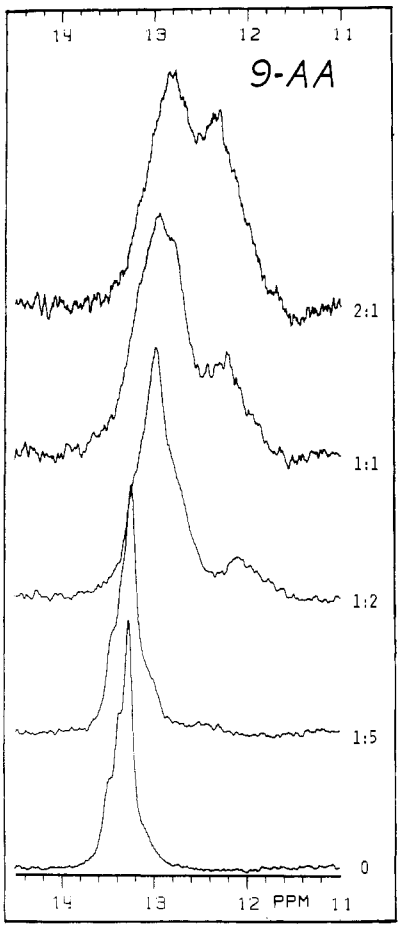

FIGURE 4: Effect of 9-aminoacridine on the low-field spectra of $d(AT)_5$·$d(AT)_5$ at 6 °C as increasing amounts of drug are added. The drug:DNA ratios are indicated on the spectra. All spectra have been adjusted to have the same peak heights.

be shifted upfield. The results with 9AA provide a clear example of how the intercalation of two chromophores affects the spectrum of $d(AT)_5$·$d(AT)_5$. Therefore, with a series of molecules containing this same chromophore, the NMR measurements provide a method for distinguishing between mono- and bisintercalation. Using the techniques described above, we carefully measured the relative intensity of the upfield shifted resonance observed in the spectrum of the 9AA complex with $d(AT)_5$·$d(AT)_5$, and this result is summarized in Table II along with similar results for other complexes.

Polymethylene Bis(acridine) Complexes (**1–5**). Figure 5 shows spectra of complexes between $d(AT)_5$·$d(AT)_5$ and the polymethylene bis(acridines) C_6, C_8, and C_{10}. In all cases at least one upfield shifted resonance can be observed, indicative of intercalative binding. For the 1:1 complexes of C_4 and C_6 (**1** and **2**) the intensity of the shifted resonance is ~24% of the total intensity, indicating that these compounds bind to the oligonucleotide with only one of the two acridine chromophores intercalated. For 1:1 complexes of C_7, C_8, and C_{10}, 40% of the low-field intensity is shifted upfield, indicating binding by bisintercalation. With the exception of compound (**2**) (Wakelin et al., 1978) these results are in agreement with viscometric studies using supercoiled DNA and the unwinding data shown in Table II.

Polyamide Bis(acridine) Complexes (**6–9**). The low-field NMR spectra of complexes of amide-linked bis(acridines) are shown in Figure 6. While there were differences in the shapes of the spectra, the fractional intensity shifted upfield in the 1:1 complexes was similar for the polymethylene bis(acridines) and amide-linked bis(acridines) with comparable chain lengths. For example, the total intensity shifted upfield (23%) on binding of compound **6** (amide-6) was approximately half of that observed for the binding of compounds **7** (amide-7a) **8** (amide-7b), and **9** (amide-8) (Figure 6), where the intensity displaced upfield corresponded to ~40% of the total intensity. These results agree with the unwinding data of Table II, except for Amd-6.

Bis(acridine) Spermine and Spermidine Complexes. Complexes of bis(acridines) joined by charged linker groups (**10** and **11**) also show upfield shifted resonances (Figure 7). For the 1:1 complex of (**11**) with $d(AT)_5$·$d(AT)_5$, two distinct and relatively sharp new peaks are observed at 12.4 and 12.8 ppm and two much broader peaks at approximately 11.9 and 12.5 ppm for the shorter spermidine-linked bis(acridine) (**10**).

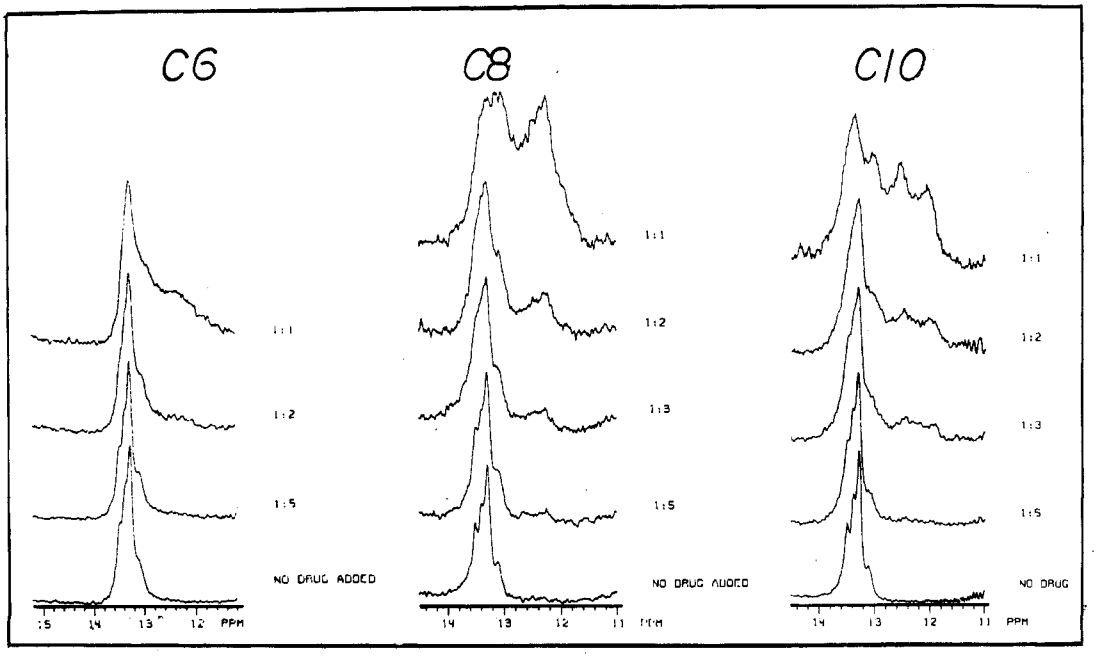

FIGURE 5: Effect of various bis(acridine) derivatives (C_6, C_8, and C_{10}) on the low-field spectrum of $d(AT)_5$·$d(AT)_5$ at 6 °C. Spectra were obtained at 300 MHz, in the correlation mode. The 4000 acquisitions were collected with a sweep time of 0.4 s, line broadened by 2–6 Hz, and then adjusted to have the same peak height.

Table II: NMR Spectral Characteristics Observed for 1:1 Complexes of $d(AT)_5$·$d(AT)_5$ with 9-Aminoacridine and 12 Bis(acridine) Derivatives

no.	compd	nature of drug shifted peaks in 1:1 complexes	type of intercalation observed by NMR	% intensity shifted[a]	unwinding angle (deg)
	9AA	1 peak, broad	monointercalator	20	16
1	C_4	1 peak, broad	monointercalator	24	17
2	C_6	1 peak, broad	monointercalator	26 ± 2[b]	30
3	C_7	1 peak, broad	bisintercalator	39	34
4	C_8	1 peak, broad	bisintercalator	38	32
5	C_{10}	2 sharp peaks	bisintercalator	41 ± 1[b]	
6	Amd-6	1 peak, broad	monointercalator	23 ± 2[b]	38
7	Amd-7	1 peak, broad	bisintercalator	38	40
8	Amd-7	1 peak, broad	bisintercalator	40	39
9	Amd-8	1 peak, broad	bisintercalator	38	35
10	spermidine	1 peak, broad	bisintercalator	45	28
11	spermine	2 sharp peaks	bisintercalator	41	
12	pyrazole	3 sharp peaks	bisintercalator	42 ± 2[b]	25

[a] ±2%. [b] Average of percent intensities obtained by two different methods.

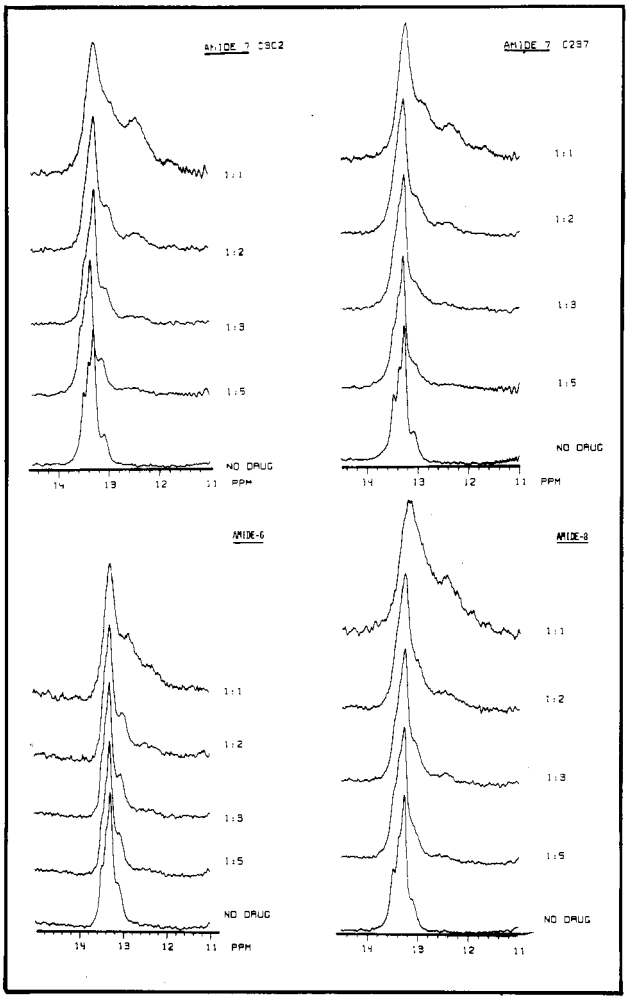

FIGURE 6: Effect of various bis(acridine) derivatives containing amide linkers on the low-field 300-MHz spectrum of $d(AT)_5$·$d(AT)_5$ at 6 °C. See caption for Figure 5 for experimental details.

The results obtained with both spermine and spermidine bis(acridine) (Figure 7) show that ~40% of the intensity is shifted upfield at a level of one drug per decamer.

Pyrazole Bis(acridine) (**12**). To minimize self-stacking and increase bisintercalative binding, compounds with increased linker chain rigidity such as the pyrazole bis(acridine) (**12**) (Denny et al., 1984a,b; L. P. G. Wakelin et al., unpublished results) have been designed that retain other desirable physicochemical properties such as aqueous solubility. The low-field spectrum of the 1:1 complex of (**12**) with $d(AT)_5$·$d(AT)_5$ (Figure 8) is similar to that obtained with C_{10} which has a comparable interligand distance (~13.7 Å). The spectrum of the 1:1 complex of **12** is different in that the highest field resonance is split (resonances at 11.9 and 11.7 ppm), indicating that the imino protons of base pairs on either side of the two chromophore are in nonequivalent environments. (At ~20 °C, this pair of resonances coalesces and forms a broad peak centered at 11.9 ppm (see below) (Assa-Munt, 1983; Assa-Munt et al., 1985). At a level of one drug per duplex, the total shifted intensity is ~41% (Figure 8) with ~12.5 ppm resonance corresponding to ~24%; those at 11.9 ppm constitute ~12% of the shifted intensity and the resonances at 11.7 ppm, ~5% of the total.

Discussion

Previous studies of ligand/DNA interaction have shown that ^{1}H NMR can positively distinguish between various modes of binding, particularly between compounds that bind by intercalation and those that bind in a groove (Feigon et al., 1984), even when random-sequence DNA is used as the substrate. For more sensitive work distinguishing between different types of intercalative binding, it seemed desirable to use a more homogeneous DNA, and in the present study, we use the synthetic DNA, $d(AT)_5$·$d(AT)_5$ as a substrate. The first issue to be addressed here concerns the possible modes of binding of the various bis(acridines) to $d(AT)_5$·$d(AT)_5$ (see Figure 1).

Mode of Binding. Bis(acridines) may bind to DNA via several different modes and each mode is predicted to have a markedly different effect on the ^{1}H NMR resonances of the imino protons in $d(AT)_5$·$d(AT)_5$. For a 1:1 drug to decamer ratio, *mono*intercalation will cause 20% of the original intensity to shift upfield. A bisintercalator that obeys the excluded site binding (model III) will shift 4 out of the 10 imino resonances upfield (40%) since each chromophore strongly perturbs the imino protons on two adjacent base pairs. If the two chromophores bind in contiguous sites (model II), only three imino resonances will be affected (30% of the intensity will shift upfield), but more importantly, the imino proton of the middle base pair sandwiched between two acridine chromophores will experience a much larger ring current shift than will the two base pairs flanking the chromophores (see Figure 1). Since an upfield shift of ~1 ppm is observed with the classical monointercalator 9AA (see Figure 4), an upfield shift as large as ~2 ppm might be expected for contiguous binding (i.e., from 13.2 to 11.2 ppm).

These various possible predictions may be compared with the experimental results obtained at 6 °C. At this temperature, the bound drugs are in slow exchange since most of the complexes exhibit well-resolved, upfield shifted resonances. If the

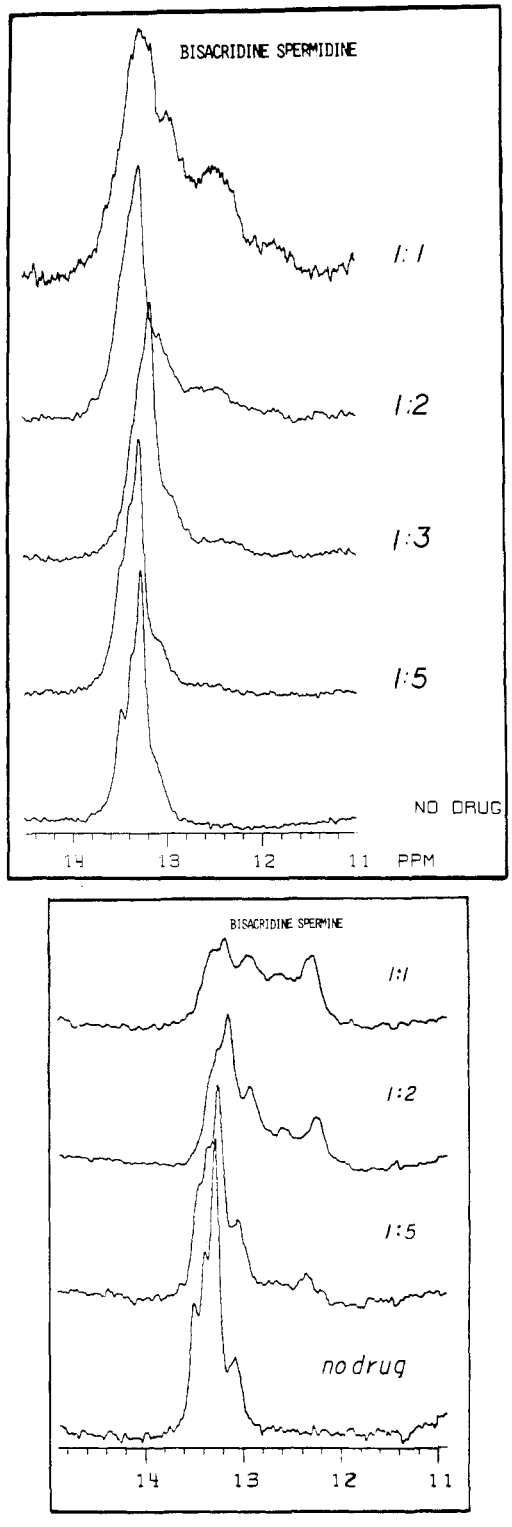

FIGURE 7: Effect of bis(acridine) spermidine (**10**) and bis(acridine) spermine (**11**) on the low-field 300-MHz spectrum of $d(AT)_5$·$d(AT)_5$ at 6 °C. The spectra of the bis(acridine) spermidine complexes have been adjusted to have the same peak height, whereas the spectra of the bis(acridine) spermine complexes have the same integrated intensities.

lifetimes of the chromophores in a specific binding site were short (less than ~2 ms), then there would have been an averaging between the shifted and unshifted low-field resonances (Feigon et al., 1982). The dynamic aspects of drug binding will be discussed in the following paper (Assa-Munt et al., 1985).

For comparison with the bis(acridines), the results obtained with the classical monointercalator (Jones et al., 1980), 9-

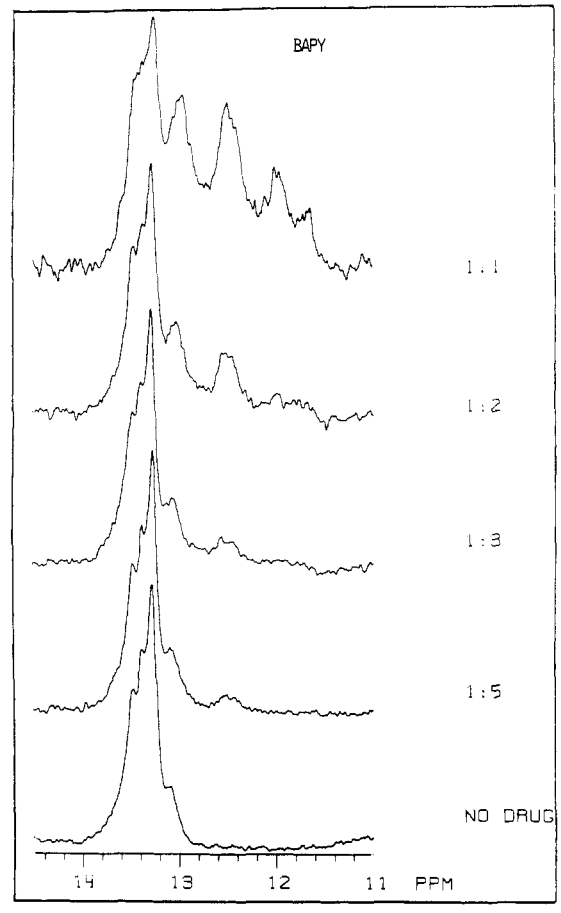

FIGURE 8: Effect of binding of the pyrazole bis(acridine) (**12**) on the low-field spectra of $d(AT_5$·$d(AT)_5$ at 6 °C.

aminoacridine, are shown in Figure 4. Binding of one 9AA per decamer duplex results in a significant (~1 ppm) upfield shift of approximately 20% of the intensity in the low-field spectrum of $d(AT)_5$·$d(AT)_5$, and binding of two 9AA per decamer [equivalent to one bis(acridine) per decamer] causes 40% of the intensity to shift upfield. This serves to reconfirm the results of earlier studies (Jones et al., 1980) which have demonstrated that this molecule binds to DNA primarily by intercalation under the salt conditions used in this investigation. The results for the various bis(acridines) are shown in Figures 5–8 and summarized in Table II. Compound **1** has a short linker chain (6.3 Å), and it can be classified as a monointercalator on the basis of the small (<24%) intensity that is shifted upfield at a level of one drug per decamer. This result is in accord with results previously obtained by using other (hydrodynamic) techniques (Wakelin et al., 1978) and the fact that the linker chain is too small to easily allow for bisintercalation, even if the two chromophores bind at adjacent sites. It is also clear from the results summarized in Table II that all of the bis(acridines) containing linker chains longer than ~10 Å can be classified as bisintercalators. At a level of one drug per decamer, all of these drugs (**3–5** and **7–12**) shift ~40% of the intensity upfield, although the exact character of the spectra varies with the nature of the linker (see below). Again the NMR results are consistent with previous hydrodynamic studies on C_7, C_8, C_{10}, bis(acridine) spermidine, and bis(acridine) spermine (Le Pecq et al., 1975; Wakelin et al., 1978) which first indicated that these drugs bind by bisintercalation. In addition, the NMR results indicate binding of these compounds by site exclusion (model III), since the upfield shifts were similar in magnitude (~1 ppm) to those observed for the monointercalator 9AA. Compounds Amd-7

and Amd-8, which had not previously been studied, are also found to bisintercalate.

The binding properties of compounds with linker chains containing six to seven atoms (9–10-Å linkers) have been the subject of some controversy. Le Pecq et al. (1975) studied a bis(acridine) with a 9.9-Å charged linker and found a behavior consistent with monointercalation. Wakelin et al. (1978), on the other hand, reported that C_6, with an 8.8-Å chain linker bisintercalates, in violation of the excluded site model. Our NMR results on Amd-6 and C_6 indicate, however, that both of these compounds bind by monointercalation. The relative intensity shifted upfield by binding of the amide-6 (23%) and C_6 (~25%) corresponds within experimental error to that expected for monointercalation, and the magnitude of the observed upfield shifts (~0.9 ppm) precludes binding at contiguous sites.

The series of 12 different bis(acridines) that we have studied here indicates that the transition from mono- to bisintercalation occurs when the chain length is increased from 8.8 to ~10 Å. Since the initial results of this study were first reported (Assa-Munt et al., 1982), other laboratories have studied the binding of bismethidium (Wilson et al., 1982) and bis(acridine) (King et al., 1982) derivatives with interchromophore separations of 8.0 and 8.8 Å. Their results also indicate that these compounds with short interchromophore separation behave as monointercalators.

This still leaves unresolved the question of why different results are obtained for compound **2** in the viscometric and NMR studies. According to a theoretical study (Shafer, 1980) of bisintercalators, high free ligand concentrations favor mono- over bisintercalation, as do high ionic strength and high temperature. These predictions have been verified (Shafer & Waring, 1980) by analysis of the binding of the mono- and bisintercalating echinomycin to DNA. In view of these observations, the discrepancy in the results obtained for compound **2** could be due to use of different salt conditions and temperature in the NMR and in the viscometric experiments, or it might be due to structural differences in the substrate $d(AT)_5 \cdot d(AT)_5$. The influence of the DNA substrate might be even more important in this regard. For example, the bisintercalator Tandem (Lee & Waring, 1978) binds more strongly to poly(dA-dT) than to calf thymus DNA, has a longer residence time on poly(dA-dT) than on calf thymus DNA, and binds to poly(dA-dT) cooperatively. This difference in the binding of Tandem to poly(dA-dT) and to calf thymus DNA was attributed to different structural properties of the two DNA substrates. More NMR and viscometric experiments using different substrates are needed in order to resolve this matter.

Effect of Linker on Character of Bisintercalation Complex. In addition to providing a method for distinguishing mono- from bisintercalative binding, the NMR technique gives more detailed information about the geometry of ligand/DNA complexes. For bis(acridines) with longer interligand separations, it is possible that bisintercalative binding could sandwich two or more base pairs between the chromophores. When a Fuller–Waring model of intercalation (Fuller & Waring, 1964) where strictly parallel stacking of the base pairs and intercalating chromophores is assumed, the minimum interligand separation necessary for the molecule to form a one-, two-, three-, or four-base pair sandwich is respectively 6.8, 10.2, 13.6, and 17.0 Å. Thus, bis(acridine) **4** with a fully extended linker chain length of 11.3 Å and **5** with one of 13.8 Å are capable of forming two- and three-base pair sandwiches, respectively. Such differences in geometry cannot easily be detected by other assays, but the NMR method makes this distinction possible for bisintercalator binding to an alternating polymer such as poly(dA-dT).

The spectrum of a complex that forms a one-base sandwich should be unique in that the interior base pair receives a very large (perhaps 2 ppm upfield shift judging from the 9AA data), and the two flanking base pairs would receive smaller (~1 ppm) shifts, leading to a total of three upfield shifted resonances per bound drug. If the linker chain permits both chromophores to bind in an equivalent manner, then a two base pair sandwich (model III, Figure 1) would result in two shifted resonances (intensity two protons each) if only one type of binding site (ApT or TpA) is occupied or four shifted resonances if both types of sites are occupied. Moreover, the interior base pairs of the sandwich would experience small additional ring current shifts from a second neighbor chromophore whereas exterior flanking base pairs would be shifted by only one chromophore. If the linker chain restricts the binding and destroys the dyad symmetry about the binding site, even more shifted resonances might be seen. In a three-base sandwich both the ApT and TpA sites are occupied, and therefore, four shifted resonances might be observed with all shifts being comparable but smaller than the two-base sandwich where the interior base pairs receive ring current shifts from two chromophores (nearest and second nearest neighbor).

It is clear from these considerations that the low-field NMR spectra alone will not necessarily provide a unique indication of exactly how the two identical chromophores of the bis(acridines) are bound to the DNA. Nevertheless, a comparison of the spectra of the 12 bis(acridine) complexes which we have studied reveals some interesting patterns. In no case did we find evidence for the formation of a single base sandwich which would have been manifested by a 30% displacement of the upfield displacement of intensity in the NMR spectra and the appearance of a single resonance shifted by as much as ~2 ppm upfield in the complex. Among those compounds that bisintercalate, as indicated by the percent intensity shifted upfield, there is an interesting correlation between chain length and the nature of the spectra. All bis(acridines) with linker chain lengths between 9.8 and 11.2 Å form complexes with spectra that are very similar to that obtained with the 2:1 complex of 9-aminoacridine in that they exhibit a broad upfield shifted peak located at about 12.3–12.4 ppm. Because of broadness, we cannot rule out the possibility that the shifted peaks contain several overlapping shifted resonances. The similarity in their spectra implies that all bind by forming two base pair sandwiches with a binding site preference similar to 9AA (model III). It is interesting then to find that the three bis(acridines) with longer linker chains (13.7 or 16.1 Å) form complexes that exhibit spectra that are different from the compounds with shorter chains and from each other. The DNA complex with **12** exhibits three upfield shifted resonances located at 12.4 (~20% of total intensity, corresponding to two protons), 11.9, and 11.7 ppm [with intensities of ~15% and 5% of the total, respectively (Figure 8)]. One possible interpretation of the spectrum is that the 11.9 and 11.7 ppm peaks arise from occupancy of the two different drug binding sites (ApT and TpA). This possibility is rejected because at elevated temperatures (27 °C) the 11.7 and 11.9 ppm peaks coalesce into a single peak at 11.9 ppm (Assa-Munt, 1983; Assa-Munt et al., 1985). Alternatively, it could be proposed that both drugs bind in the same type of site, but in two different, interconvertible ways. We favor this latter interpretation since the 11.9 ppm resonance is the slowest one to

relax at 27 °C, consistent with its assignment to the interior two base pairs which are sandwiched between the two chromophores. The 12.4 ppm resonances relax faster and, therefore, are reasonably assigned to the exterior two base pairs that flank the two chromophores (Assa-Munt, 1983; Assa-Munt et al., 1985).

The spectrum of the bis(acridine) spermine complex could be interpreted in several ways. The fact that the most upfield shifted resonance is located at the same position as that observed with 9AA complex with $d(AT)_5$·$d(AT)_5$ could imply that one chromophore binds in the normal preferred site and the second chromophore binds in a second site located three base pairs away. This would account for the relatively small upfield shifts of the $d(AT)_5$·$d(AT)_5$ resonances (no second neighbor effect), but it would not account for the fact that the 12.4 ppm resonance is especially stable with respect to thermal denaturation (Assa-Munt, 1983; Assa-Munt et al., 1985). Alternatively, the two chromophores may bind at equivalent sites (ApT or TpA) but not in an equivalent manner, because interactions between the charged linker chain and the oppositely charged phosphate backbone restrict the exact manner in which the two acridine residues intercalate and reduce the magnitude of the ring current shifts. (Note that a smaller ring current shift on the thymine imino resonance does not necessarily correspond to poorer stacking of the acridine and surrounding base pairs). The most shifted (12.4 ppm) resonances would be attributed to the two interior base pairs whereas the 12.8 ppm resonances could arise from the exterior pairs (Figure 1, model III) in the bis(acridine) spermine complex. In support of this interpretation, we note that Dickerson et al. (1983) find that spermine induces a bend in the dodecamer $d(CGCGATATCGCG)_2$. We also favor this latter interpretation because the most upfield shifted resonance is so stable with respect to thermally induced exchange with solvent protons, while the resonance at 12.8 is labile, even at 7 °C (Assa-Munt, 1983; Assa-Munt et al., 1985).

While we are not able to deduce the structure of these complexes using just the low-field spectra, the present results reveal that there are important variations that depend on both the length and nature of the linker (neutral, charged, flexible, or rigid). Determination of the structural details will require use of additional one- and two-dimensional NMR techniques. In the following paper (Assa-Munt et al., 1985), we have examined the effect of temperature on the spectra of the bis(acridine) complexes and carried out spin–lattice relaxation measurements (Assa-Munt et al., 1982). The results of these studies provide insight into the dynamic aspects of the complexes as well as additional information about the nature of the complexes.

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REFERENCES

Albert, A. (1966) in *The Acridines*, St. Martins Press, New York.

Assa-Munt, N. (1983) Thesis, University of California, San Diego.

Assa-Munt, N., Denny, W. A., Leupin, W., & Kearns, D. R. (1982) *Biophys. J. 37*, 290a.

Assa-Munt, N., Leupin, W., Denny, W. A., & Kearns, D. R. (1985) *Biochemistry* (following paper in this issue).

Atwell, G. J., Leupin, W., Twigden, S. J., & Denny, W. A. (1983) *J. Am. Chem. Soc. 105*, 2913–2914.

Baguley, B. C., Denny, W. A., Atwell, G. J., & Cain, B. F. (1981) *J. Med. Chem. 24*, 520–525.

Bauer, W., & Vinograd, J. (1970) *J. Mol. Biol. 47*, 419–435.

Becker, M. M., & Dervan, P. (1979) *J. Am. Chem. Soc. 101*, 3664–3666.

Berman, H. M., & Young, P. R. (1981) *Annu. Rev. Biophys. Bioeng. 10*, 87–114.

Cain, B. F., Baguley, B. C., & Denny, W. A. (1978) *J. Med. Chem. 21*, 658–668.

Canellakis, E. S., Bono, V., Bellantone, R. A., Krakow, J. S., Fico, R. M., & Schulz, R. A. (1976) *Biochim. Biophys. Acta 418*, 300–314.

Capelle, N., Barbet, J., Dessen, P., Blanquet, S., Roques, B. P., & Le Pecq, J.-B. (1979) *Biochemistry 18*, 3354–3362.

Chen, T. K., Fico, R., & Canellakis, E. S. (1978) *J. Med. Chem. 21*, 868–874.

Crothers, D. M. (1968) *Biopolymers 6*, 575–584.

Delbarre, A., Gourevitch, M. I., Gaugain, B., Le Pecq, J. B., & Roques, B. P. (1983) *Nucleic Acids Res. 11*, 4467–4482.

Denny, W. A., Cain, B. F., Atwell, G. J., Hansch, C., Panthanickal, A., & Leo, A. (1982a) *J. Med. Chem. 25*, 276–315.

Denny, W. A., Leupin, W., & Kearns, D. R. (1982b) *Helv. Chim. Acta 65*, 2372–2393.

Denny, W. A., Atwell, G. ., Baguley, B. C., Stewart, G. M., Twigden, S. J. (1984a) *J. Med. Chem.* (submitted for publication).

Denny, W. A., Baguley, B. C., Cain, B. F., & Waring, Mm. J. (1984b) in *Molecular Aspects of Anticancer Drug Action* (Neidle, S., & Waring, M. J., Eds.) pp 1–34, Macmillan, London.

Dickerson, R. E., Kopka, M. L., & Pjura, P. (1983) *J. Biomol. Struct. Dynam. 1*, 755–771.

Dougherty, G. (1982) *Comments Mol. Cell. Biophys. 1*, 337–354.

Feigon, J., Leupin, W., Denny, W. A., & Kearns, D. R. (1982) *Nucleic Acids Res. 10*, 749–762.

Feigon, J., Denny, W. A., Leupin, W., & Kearns, D. R. (1984) *J. Med. Chem. 27*, 450.

Fico, R. M., Chen, T. K., & Canellakis, E. S. (1977) *Science (Washington, D.C.) 198*, 53–56.

Fuller, W., & Waring, M. J. (1964) *Ber. Bunsenges. Phys. Chem. 68*, 805–808.

Gaugain, B., Barbet, J., Oberlin, R., Roques, B. P., & Le Pecq, J. B. (1978) *Biochemistry 17*, 5071–5078.

Jones, R. L., Lanier, A. C., Keel, R. A., & Wilson, W. D. (1980) *Nucleic Acids Res. 8*, 1613–1624.

Kearns, D. R. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 477–523.

King, H. D., Wilson, W. D., & Gabbay, E. J. (1982) *Biochemistry 21*, 4982–4989.

Kuhlmann, K. F., & Mosher, C. W. (1981) *J. Med. Chem. 24*, 1333–1337.

Lee, J. S., & Waring, M. J. (1978) *Biochem. J. 173*, 129–144.

Le Pecq, J.-B., Xuong, N.-D., Gosse, C., & Paoletti, C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 5078–5082.

Le Pecq, J.-B., Le Bret, M., Barbet, J., & Roques, B. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2915–2919.

Lerman, L. S. (1961) *J. Mol. Biol. 3*, 18–30.

Lown, J. W., Gunn, B. C., Chang, R.-Y., Majumdar, K. C., & Lee, J. S. (1978) *Can. J. Biochem. 56*, 1006–1015.

McGhee, J. D., & von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469–489.
Patel, D. J., & Hilbers, C. W. (1975) *Biochemistry 14*, 2651–2656.
Shafer, R. H. (1980) *Biopolymers 19*, 419–430.
Shafer, R. H., & Waring, M. J. (1980) *Biopolymers 19*, 431–443.
Sobell, H. M. (1980) in *Nucleic Acid Geometry and Dynamics* (Sarma, R. H., Ed.) Pergamon Press, New York.
Wakelin, L. P. G., & Waring, M. J. (1976) *Biochem. J. 157*, 721–740.
Wakelin, L. P. G., Romanos, M., Chen, T. K., Glaubiger, D., Canellakis, E. S., & Waring, M. J. (1978) *Biochemistry 17*, 5057–5063.
Waring, M. J. (1981) *Annu. Rev. Biochem. 50*, 159–192.
Wilson, W. D., & Jones, R. L. (1982) in *Intercalation Chemistry*, Academic Press, New York.
Wilson, W. D., Keel, R. A., Jones, R. L., & Mosher, C. W. (1982) *Nucleic Acids Res. 10*, 4093–4106.
Wright, R. G. McR., Wakelin, L. P. G., Fieldes, A., Acheson, R. M., & Waring, M. J. (1980) *Biochemistry 19*, 5825–5836.

^{1}H NMR Study of the Binding of Bis(acridines) to d(AT)$_5$·d(AT)$_5$. 2. Dynamic Aspects†

Nuria Assa-Munt,‡ Werner Leupin,§ William A. Denny,‖ and David R. Kearns*
Department of Chemistry, University of California—San Diego, La Jolla, California 92093
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ABSTRACT: Measurements of the ^{1}H NMR spectra and relaxation rates were used to study the dynamic properties of 9-aminoacridine (9AA) and four bis(acridine) complexes with d(AT)$_5$·d(AT)$_5$. The behavior of the 9AA (monointercalator) and that of C_8 (bisintercalator containing an eight-carbon atom linker chain) are entirely similar. For both compounds, the lifetime of the drug in a particular binding site is 2–3 ms at ~20 °C, and neither affects the A·T base pair opening rates. The complex with C_{10} (bisintercalator containing a 10-carbon atom linker chain) is slightly more stable than the C_8 complex since its estimated binding site lifetime is 5–10 ms at 29 °C. Base pairs adjacent to the bound C_{10} are destabilized, relative to free d(AT)$_5$·d(AT)$_5$, but other base pairs in the C_{10} complex are little affected. Bis(acridine) pyrazole (BAPY) and bis(acridine) spermine (BAS) considerably stabilize those base pairs that are sandwiched between the two acridine chromophores, but in the BAS complex proton exchange from the two flanking base pairs appears to be accelerated, relative to free d(AT)$_5$·d(AT)$_5$. The lifetime of these drugs in specific binding sites is too long (>10 ms) to be manifested in increased line widths, at least up to 41 °C. An important conclusion from this study is that certain bisintercalators rapidly migrate along DNA, despite having large binding constants ($K > 10^6$ M^{-1}). For C_8 and C_{10} complexes, migration rates are little different from those deduced for 9AA. The rigid linker chain in BAPY and the charge interactions in BAS retard migration of these two bisintercalators. These results provide new parameters that are useful in understanding the biochemical and biological properties of these and other bisintercalating drugs.

In an effort to design rationally drugs with enhanced chemotherapeutic properties, many compounds have been screened for biological activity, and their activity has been compared with a variety of measurable physical properties of the drugs (Le Pecq et al., 1974; Fink et al., 1980; Baguley et al., 1981; Feigon et al., 1984). For certain classes of compounds, these studies clearly show that DNA binding strength is one of the parameters important to biological activity, and therefore, to obtain compounds with greater binding strength, a number of bifunctional intercalating compounds containing two chromophores joined by a linker chain were synthesized (Le Pecq et al., 1974, 1975; Gaugain et al., 1978; Mosher & Capelle, 1979; Ikeda & Dervan, 1982). In addition to stronger binding, these compounds should exhibit enhanced sequence specificity. Moreover, since there is some evidence that biological activity is better correlated with a low dissociation rate from the DNA (Muller & Crothers, 1968), the potential bisintercalators were expected to be better because they also should dissociate more slowly from the DNA (Capelle et al., 1979). Among the potential bisintercalating compounds that have been prepared, the bis(acridines) are the group most thoroughly studied (Chen et al., 1978; Lown et al., 1978; Capelle et al., 1979; Wright et al., 1980; King et al., 1982), and while many do exhibit enhanced binding and slow dissociation kinetics, they have not shown the greatly enhanced biological activity that was anticipated (Denny et al., 1984a). One missing factor, in addition to binding strength and dissociation rates, that might be important to the biological activity of a drug is the rate at which it migrates between binding sites, *without* dissociating from the DNA. Unfortunately, little is known about the rate at which drugs migrate between sites along the DNA before reintercalating or dissociating com-

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‡ Present address: The Salk Institute, La Jolla, CA 92138.
§ Permanent address: Institut fur Molekularbiologie und Biophysik, ETH-Honggerberg, CH-8093 Zurich, Switzerland.
‖ Permanent address: Cancer Chemotherapy Research Laboratory, University of Auckland School of Medicine, Private Bag, Auckland, New Zealand.

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