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

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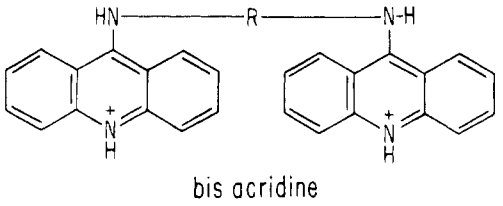
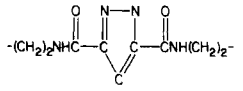
Table I: Structure and Nomenclature of Bis(acridine) Compounds




bis acridine

| nomenclature | atoms in linker chain | linker | $R$ = (Å)[a] |
|---|---|---|---|
| $C_8$ | 8 | $-(CH_2)_8-$ | 11.3 |
| $C_{10}$ | 10 | $-(CH_2)_{10}-$ | 13.8 |
| spermine (BAS) | 12 | $-(CH_2)_3NH(CH_2)_4NH(CH_2)_3-$ | 16.1 |
| pyrazole (BAPY) | 11 |  | 13.7 |

[a] N to N distance.

pletely. Since the lifetimes of the drugs in specific binding sites may affect their biological activity, it is important to have measurements of these quantities for various DNA binding drugs. Another aspect of drug–DNA interactions that has received little attention concerns the effect that the drugs have on base pair dynamics (opening and closing kinetics) (Patel & Canuel, 1979; Assa-Munt et al, 1982b; Pardi et al., 1983). While many drugs stabilize the DNA duplex with respect to thermal denaturation (Capelle et al., 1979), little is known about the effect these drugs have on the local base pairing structure and dynamics at the binding sites.

In the preceding paper (Assa-Munt et al., 1985), we used $^1$H NMR to investigate the modes of binding of various bis(acridines) to the decanucleotide d(AT)$_5$·d(AT)$_5$. In the present study $^1$H NMR spectra and relaxation measurements at different temperatures are used to explore dynamic aspects of the binding of four bis(acridines) (see Table I) all of which have been shown to bind to d(AT)$_5$·d(AT)$_5$ by bisintercalation (Assa-Munt et al., 1985). For purposes of comparison, we have also studied the NMR behavior of free d(AT)$_5$·d(AT)$_5$ and its complex with the monointercalator 9-aminoacridine (9AA).

The NMR measurements reported here are specifically concerned with the low-field resonances from the hydrogen-bonded imino protons of thymine. These resonances are particularly useful in drug binding studies since they (1) are located in a spectral region well removed from all other DNA and drug resonances, (2) permit drug binding at specific sites (intercalation) to be monitored, (3) can be used to monitor the base pair opening rates and the effect of the drugs on these rates, and (4) are sensitive to the drug binding kinetics.

## Materials and Methods

*Materials.* The preparation of d(AT)$_5$·d(AT)$_5$ is described by Denny et al. (1982b). The bis(acridines) were prepared in the Cancer Chemotherapy Research Laboratory, University of Auckland School of Medicine, by methods reported (Chen et al., 1978; Denny et al., 1984a).

*Methods.* NMR spectra were carried out either on a Varian HR 300 MHz spectrometer modified by Dr. T. A. Early to operate as a Fourier transform spectrometer or on a home-built 360-MHz spectrometer constructed by Dr. John M. Wright. All spin–lattice and spin–spin relaxation studies were made at 360 MHz in buffered $H_2O$ solutions by using the modified time-shared Redfield 214 pulse sequence as described by Wright et al. (1981). Typically ~500 scans were accumulated ($D_2$ = 2 s) and line broadened by 5 Hz. The preparation of the drug–DNA complexes for the NMR measurements is described in the preceding paper (Assa-Munt et al., 1985). Line widths of resonances in the low-field spectra were determined by using the Nicolet program to simulate spectra.

## Results

The spectrum of a drug–DNA complex will be affected in several ways by an increase in temperature. First, there will be an increase in the rate of exchange of the imino protons with water protons that will lead to broadening and eventual disappearance of low-field resonances. Therefore, by comparing the spectra of the free DNA and the drug–DNA complex at various temperatures, one can obtain information about the relative stability of base pairs in free DNA as compared to the DNA in the drug–DNA complex. Broadening of resonances in the drug–DNA complexes can also occur because of the finite lifetime, $\tau$, of the intercalated drugs at specific binding sites. If this extra broadening contribution to the line width can be estimated, it is possible to determine $\tau$ values. These two different sources of broadening can be distinguished from each other by measuring the spin–lattice and spin–spin relaxation rates. In the present study, we have carried out measurements of the spectra as a function of temperature and of spin–lattice and spin–spin relaxation rates for free d(AT)$_5$·d(AT)$_5$ and its complexes with the various drugs at various temperatures.

### *Temperature Dependence of Spectra*

*d(AT)$_5$·d(AT)$_5$.* The low-field spectra of d(AT)$_5$·d(AT)$_5$ duplex at two temperatures are shown in Figure 1. Resonances A–E arise from the five pairs of nonidentical imino protons. As the temperature increases from 5 to 23 °C, peaks A, B, and E broaden and disappear, and on the basis of the order in which they broaden, we have tentatively assigned the resonances to the following base pairs: A = 2, B = 3, C = 4, D = 5, and E = 1. Resonance E is attributed to terminal base pair 1 since it broadens first. At 23 °C, the intensity of the other resonances also decreases (data not shown) because of the rapid exchange of imino protons with water. Deconvolution of these spectra using the Nicolet 1180E software indicates that at 18 °C only ~6 of the original 10 are still present.

*9-Aminoacridine (9AA).* Figure 2 shows the effect of temperature on a 9-aminoacridine–d(AT)$_5$·d(AT)$_5$ complex at a ratio of 2.2 drugs per 10 base pairs. This ratio was chosen for comparison with complexes of one bis(acridine) per 10 base pairs. At 6 °C, a separate upfield shifted peak at 12.3 ppm (representing ~40% of the total integrated intensity) is visible, and the original "unperturbed" imino resonances have experienced a small shift, from ~13.3 to ~12.9 ppm. At 15 °C, the upfield shifted peak arising from the imino protons of base pairs at the drug binding site has broadened substantially, and by 17 °C it has coalesced with the original peak from the unshifted (AT) imino protons. Note that the coalesced peak that appears at 12.7 ppm is located midway between the main peak in the original unperturbed spectrum (13.3 ppm) and the most shifted resonance (12.3 ppm) in the low temperature spectrum of the complex.

*$C_8$.* The effect of temperature on the 300-MHz spectra of a 1:1 complex of d(AT)$_5$·d(AT)$_5$ with a bisintercalator containing a linker chain with eight methylene groups ($C_8$) is shown in Figure 3. In many regards the properties of the $C_8$ and the 9AA complexes are similar. This compound is obviously in slow exchange at 5 °C, but by 15 °C the upfield shifted resonances (corresponding to ~40% of the total in-

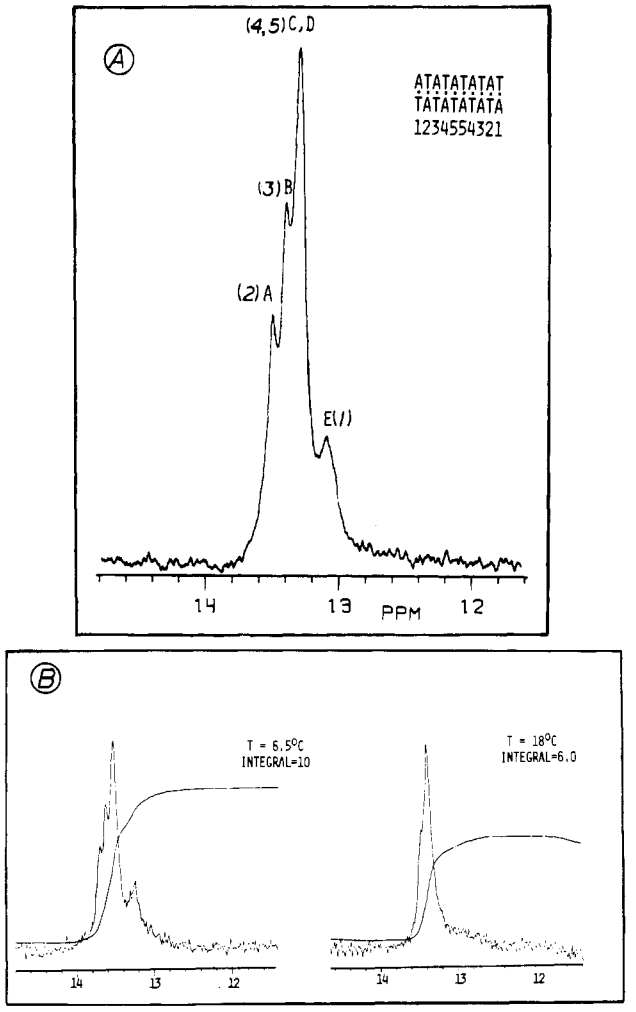



FIGURE 1: (A) Low-field spectrum of $d(AT)_5$·$d(AT)_5$ with assignment of the resonances indicated. (B) A comparison of the 6.5 and 18 °C spectra of $d(AT)_5$·$d(AT)_5$ showing a decrease in the integrated intensity from 10 to ~6 protons.

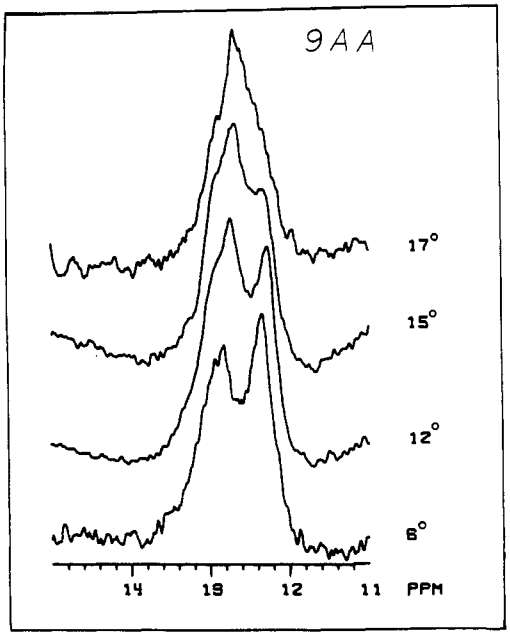



FIGURE 2: Effect of temperature on the low-field spectra of 2:1 9AA–$d(AT)_5$·$d(AT)_5$ complex at 300 MHz. Temperatures are indicated on figure.

tensity) broaden and coalesce, and by 21 °C some loss of intensity, because of exchange with water, was evident. Note

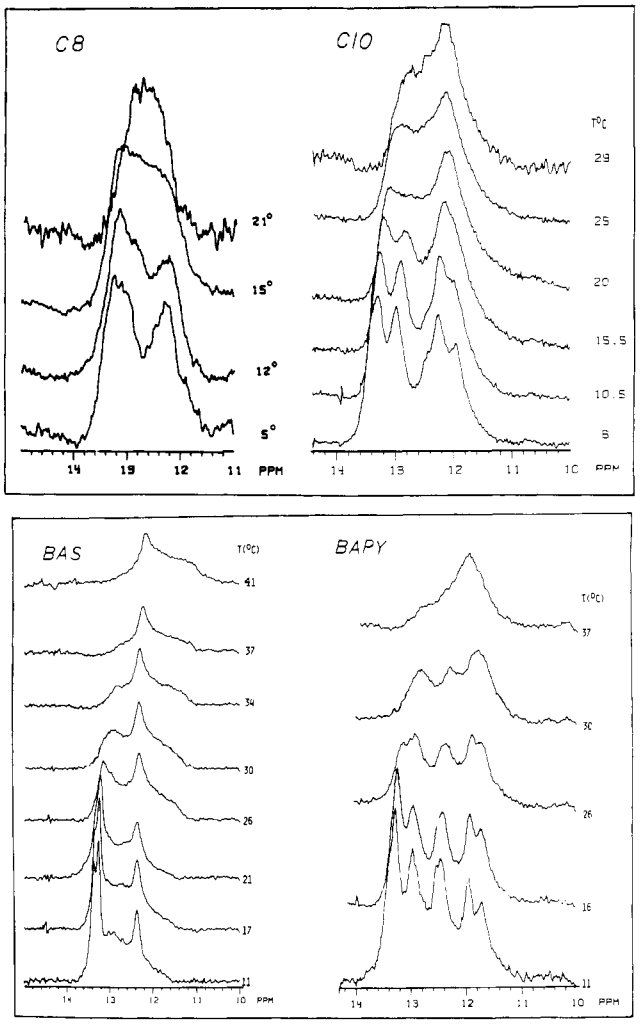



FIGURE 3: Effect of temperature on the low-field spectra of 1:1 complexes of $C_8$, $C_{10}$, bis(acridine) spermine (BAS), and bis(acridine) pyrazole (BAPY) with $d(AT)_5$·$d(AT)_5$ at a ratio of one drug per decamer.

that the coalesced peak in the 21 °C spectra appears at 12.6 ppm, whereas in the free $d(AT)_5$·$d(AT)_5$ spectrum the peaks remain at 13.2 ppm at 22 °C.

*$C_{10}$.* Figure 3 shows the spectra of a 1:1 complex of $C_{10}$ with $d(AT)_5$·$d(AT)_5$ at a level of one drug per 10 base pairs. Addition of this drug causes upfield shifts of the imino proton resonances of bases adjacent to the binding site and results in the appearance of two distinct new peaks upfield in the 6 °C spectrum at ~12.5 and 11.5 ppm. On warming to 15.5 °C, these two most upfield shifted peaks coalesce into a single broader peak, and by 20 °C all resonances (unshifted and drug shifted) are noticeably broadened. At 29 °C some loss of intensity in the "unshifted" resonances is evident, and the two peaks are beginning to coalesce.

*Bis(acridine) Spermine (BAS).* The temperature of the spectrum of a 1:1 complex of the bis(acridine) spermine–$d(AT)_5$·$d(AT)_5$ complex is shown in Figure 3. On heating from 11 to 26 °C, resonances arising from the imino protons in the complex experience differential broadening because of exchange with $H_2O$ (see discussion of relaxation rates). A resonance at 12.95 ppm is the first to broaden and disappear. Those resonances that are more upfield shifted due to chromophore binding are also more stable, and the most upfield shifted resonance (at ~12.4 ppm) is the most stable resonance. Comparison of the 6 (see relaxation data) and 20 °C spectra of the bis(acridine) spermine–$d(AT)_5$·$d(AT)_5$ complex shows that 8 out of the initial 10 imino protons are still present at

Table II: Spin–Lattice ($R_1$) and Spin–Spin ($R_2$) Relaxation Rates of the Imino Protons in Free d(AT)$_5$·d(AT)$_5$ and in a 1:1 Complex of BAS and d(AT)$_5$·d(AT)$_5$ at 6 ± 1 °C

| | | resonance position (ppm) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $R_{1,2}$ ($s^{-1}$) | 13.44 | 13.38 | 13.26 | 13.03 | 13.04 | 12.7 | 12.4 |
| free d(AT)$_5$·d(AT)$_5$ | $R_1$ | 6.25 | 4.2 ± 0.5 | 4.5 ± 0.5 | 78 ± 5 | | | |
| | $R_2$ | 45 ± 5 | 40 ± 5 | 55 ± 2 | 66 ± 5 | | | |
| BAS complex (1:1) | $R_1$ | | 20 ± 3 | 3 ± 2 | | 42 ± 5 | 79 ± 3 | 4 ± 1 |
| | $R_2$ | | 76 ± 7 | 72 ± 5 | | 125 ± 8 | 133 ± 6 | 87 ± 5 |

20 °C (not shown) whereas with free d(AT)$_5$·d(AT)$_5$ only ~6 imino protons are observed at 20 °C. By 26 °C, resonances at ~13.4 ppm have broadened considerably. Bis(acridine) spermine obviously stabilizes some base pairs of d(AT)$_5$·d(AT)$_5$ with respect to exchange since imino proton resonances are still measurable at 41 °C in the bis(acridine) spermine–d(AT)$_5$·d(AT)$_5$ complex whereas for free d(AT)$_5$·d(AT)$_5$ and for the $C_8$ and $C_{10}$ bisintercalator–d(AT)$_5$·d(AT)$_5$ complexes the resonances have broadened and disappeared at lower temperatures (see Figure 3). The high temperature spectra are unusual, however, in that a broad new resonance appears at ~11.5 ppm. This could arise from an interaction between thymine and the spermine linker chain; however, not enough information is available at this point to define the interactions involved.

*Bis(acridine) Pyrazole (BAPY).* The complex formed by BAPY and d(AT)$_5$·d(AT)$_5$ at one drug per 10 base pairs was studied at different temperatures with the results shown in Figure 3. At low temperatures many drug-shifted resonances are observed, but by 30 °C, broadening and loss of intensity of the unshifted drug resonances starts to occur. At 37 °C, only the drug-shifted imino proton resonances are still present.

From the data presented above, it is clear that for all bis-intercalator–DNA complexes studied, those imino proton resonances that are most shifted by the drug chromophores are still observable at elevated temperatures where unshifted resonances have started to broaden due to exchange. For complexes with bis(acridine) spermine or BAPY, drug-shifted resonances can be observed at 37 °C, whereas for the free d(AT)$_5$·d(AT)$_5$ decamer, loss of intensity, because of exchange, is evident at 21 °C; by 31 °C the low-field intensity is reduced to half that observed at 21 °C.

### *Relaxation Studies*

*d(AT)$_5$·d(AT)$_5$.* To aid the interpretation of the relaxation properties of drug–DNA complexes, relaxation measurements were first carried out on free d(AT)$_5$·d(AT)$_5$ at 6, 20, and 27 °C. At 6 °C, resonances from all imino protons in d(AT)$_5$·d(AT)$_5$ can be observed, but they exhibit different relaxation rates (Figure 4). Decay curves for each resonance are shown in Figure 4 and Table II summarizes the spin–lattice ($R_1$) and spin–spin ($R_2$) relaxation rates for the different imino protons at 6 °C.

Nonexponentiality of the spin–lattice recovery rate, most apparent at low temperatures where dipolar interactions dominate the relaxation, arises, in part, from superimposing resonances recovering at different rates (Granot, 1982) and from spin-diffusion effects (Assa-Munt et al., 1984). Some association of the decamers at 6 °C cannot be completely ruled out, and this could lead to an initial fast component in the decay. For this reason, relaxation rates in d(AT)$_5$·d(AT)$_5$ were calculated by using decay points measured after the first ~10 ms.

In free d(AT)$_5$·d(AT)$_5$ the resonances at 13.03 and 13.46 ppm are the first to recover, while resonances at 13.27 ppm relax with the slowest rate. On the basis of these experiments and the temperature data discussed above, the most rapidly

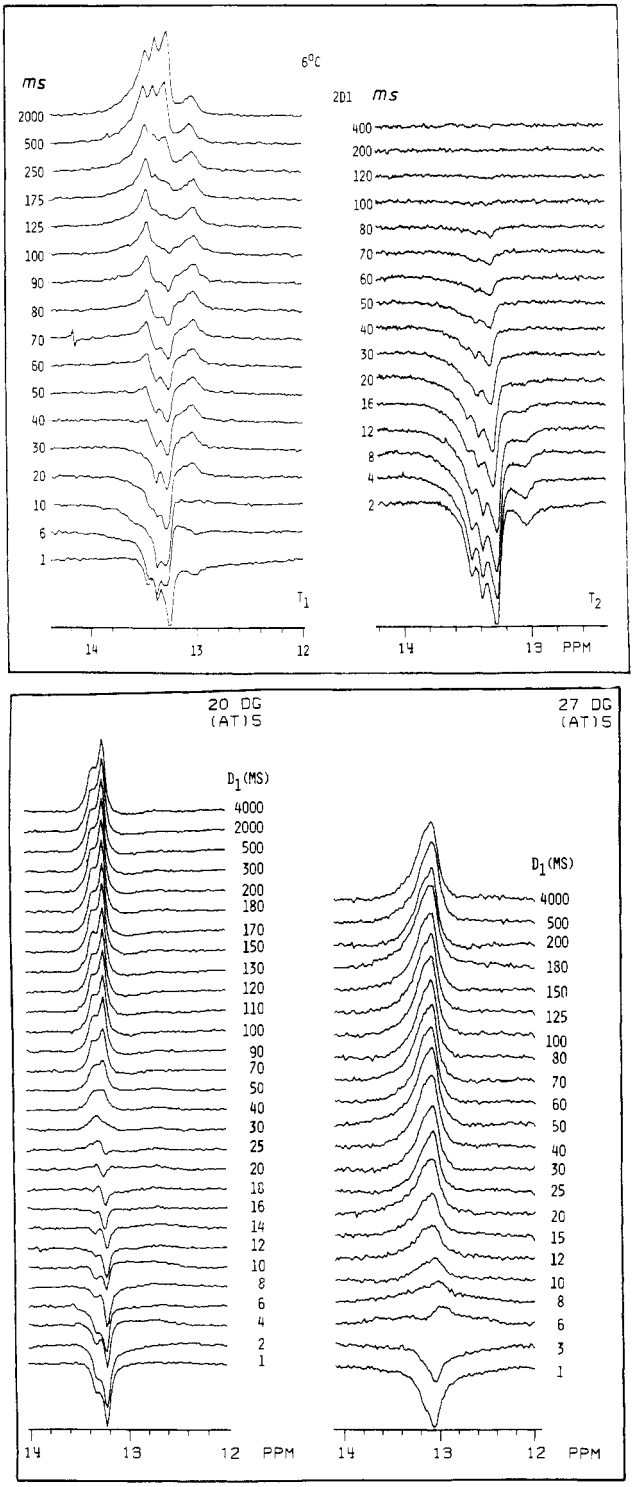



FIGURE 4: Experimental low-field spectra obtained from relaxation measurement on d(AT)$_5$·d(AT)$_5$ at 6, 20, and 27 °C at 360 MHz.

relaxing resonance, at 13.03 ppm, is assigned to the terminal base pair imino protons (base pair 1) since terminal base pairs (especially A·T base pairs) exhibit faster exchange of their

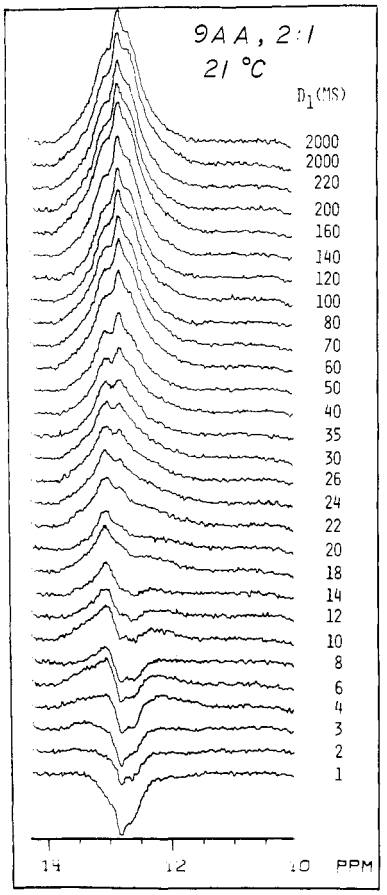



FIGURE 5: Experimental spectra obtained from a longitudinal relaxation measurement on a 2.2:1 9AA–d(AT)$_5$·d(AT)$_5$ complex at 21 °C.

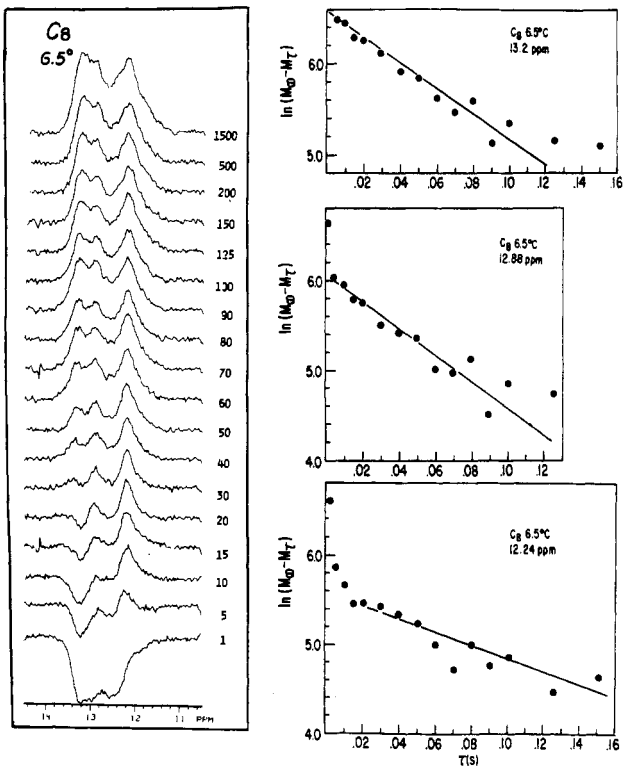



FIGURE 6: Experimental spectra obtained from longitudinal relaxation measurements at 6.5 °C on a 1:1 C$_8$–d(AT)$_5$·d(AT)$_5$ complex (top) are presented along with semilog plots of longitudinal relaxation for imino resonance at 6.5 °C (bottom).

imino protons with solvent than do interior base pairs (Patel & Hilbers, 1975). Correspondingly, the most slowly relaxing resonances (at 13.26 ppm) are assigned to the two inner base pairs of the d(AT)$_5$·d(AT)$_5$ decamer (base pairs 5 and 4); resonances at 13.46 and 13.37 ppm are assigned to the second and third base pair from the terminus (base pairs 2 and 3). At higher temperatures, exchange with solvent dominates the dipolar contribution to the spin–lattice relaxation rate, and $R_1$ thus becomes a measure of the rate of imino proton exchange with solvent (Teitelbaum & Englander, 1975a,b; Johnston & Redfield, 1977; Early et al., 1981a,b).

Experimental data obtained at 20 and 27 °C are shown in Figure 4. At 20 °C, only resonances arising from the interior base pairs can be monitored since protons in the terminal and next-to-terminal base pairs are in fast exchange with solvent and have disappeared (Figure 4). By 27 °C, only a broad, rapidly relaxing ($R_1 \simeq 80$ s$^{-1}$) resonance can be monitored. Relaxation rates for the "innermost", i.e., the most stable d(AT)$_5$·d(AT)$_5$, resonance at different temperatures are presented in Table III.

*9AA.* Spin–lattice relaxation experiments were performed on the 2:1 9AA–d(AT)$_5$·d(AT)$_5$ complex at the coalescence temperature (~21 °C) with the results shown in Figure 5 and in Table III. The lowest field resonance, at 12.99 ppm, relaxes fast (87 s$^{-1}$), but the main resonance intensity (resonances at ~12.63 and 12.8 ppm) recovers with a rate of ~28 ± 2 s$^{-1}$, which is comparable to the rate (25 ± 3 s$^{-1}$) observed for interior base pairs in free d(AT)$_5$·d(AT)$_5$ at this temperature.

*$C_8$.* Figure 6 shows the spin–lattice relaxation of the C8–d(AT)$_5$·d(AT)$_5$ complex at 6 °C. The "original", unperturbed resonances at 13.2 ppm relax at a rate of ~15 s$^{-1}$ whereas the separate broad upfield shifted peak at ~12.3 ppm exhibits a fast (~100 s$^{-1}$) and a slow (~8 s$^{-1}$) decay component. The fact that resonances at 13.2 ppm decay with a slow rate constant proves that the fast rates (~100 s$^{-1}$) observed for the drug-shifted resonances are not due to formation of aggregates. We, therefore, conclude that two types of drug-shifted imino resonances are present at 12.3 ppm. At 21 °C, the drug-shifted imino resonances and the unperturbed imino resonances have coalesced (Figure 3). Although recovery of the lower field side of this broad peak is slightly faster, the relaxation rates of resonances at ~12.4 (~60 s$^{-1}$) and 12.9 ppm (~70 s$^{-1}$) are comparable (Table III). These proton exchange rates are much too slow to account for the substantial broadening of the resonances which is observed (see Discussion).

*$C_{10}$ Complexes.* Most resonances in the 6 °C spectrum of the $C_{10}$ complex exhibit two-component decay curves with rate constants of 6–9 and 14–18 s$^{-1}$ (see Table III). The decays of the 13.0 ppm and the drug-shifted 12.26 ppm resonances are different, however, in that they exhibit a fast initial decay (42 and 50 s$^{-1}$, respectively). The behavior of the 13.0 ppm resonance suggests that it is due to a terminal base pair that has been slightly stabilized in the drug complex, since, in free d(AT)$_5$·d(AT)$_5$, the end base pair decays with a rate of ~80 s$^{-1}$ (see Table III). The fast decay of the drug-shifted resonances at 12.26 ppm indicates that the binding of the drug has *destabilized* this base pair, with respect to proton exchange with the solvent. The slow component in the decay at 12.26 ppm can be attributed to contributions from overlapping neighboring resonances that relax more slowly.

At 20 °C, there are changes in the spectrum that indicate some loss of resonances and/or broadening and shifting of peaks (Figure 3). Thus, the 13.4 ppm peak broadens and shifts upfield to 13.1 ppm and the most upfield shifted drug resonance shifts *downfield* from 11.9 to 12.1–12.2 ppm. The resonances at 12.26 and 13.0 ppm (which relax rapidly at 6 °C) are probably decaying too fast to be seen in the 20 °C

Table III: Spin–Lattice Relaxation Rates for $d(AT)_5 \cdot d(AT)_5$ and Its Complexes with 9AA, $C_8$, $C_{10}$, Bis(acridine) Spermine, and Bis(acridine) Pyrazole

| complex | $T$ (°C) ± 1 | relaxation rates, $R_1$ ($s^{-1}$)[a] unshifted drug resonances | coalesced resonances | shifted drug resonances |
|---|---|---|---|---|
| free $d(AT)_5 \cdot d(AT)_5$[e] | 6 | 4.2 (13.38)[b] | | |
| | | 4.5 (13.26) | | |
| | 21 | 28 (13.32) | | |
| | | 21 (13.23) | | |
| | 27 | 80 (13.08) | | |
| 9AA–$d(AT)_5 \cdot d(AT)_5$[c] | 21 | | 87 (12.99) | |
| | | | 24 (12.49) | |
| | | | 30 (12.63) | |
| $C_8$–$d(AT)_5 \cdot d(AT)_5$ | 6.5 | 14 (13.2) | | 15 (12.88) |
| | | | | 100, 7.5 (12.28) |
| | 21 | | 71 (12.91) | |
| | | | 58 (12.4) | |
| $C_{10}$–$d(AT)_5 \cdot d(AT)_5$ | 6 | 6, 16 (13.4)[d] | | 18, 9 (12.50)[d] |
| | | 8, 16 (13.3) | | 50, 5 (12.26) |
| | | 42 (13.0) | | 14, 7 (11.9) |
| | 20 | 30 (13.1) | | 29 (12.6) |
| | | | | 22 (12.1) |
| | 32.5 | | | 83 (12.7) |
| | | | | 91 (12.2) |
| bis(acridine) spermine–$d(AT)_5 \cdot d(AT)_5$[e] (BAS) | 7 | 20 (13.38) | | 79 (12.7) |
| | | 3 (13.26) | | 4 (12.4) |
| | | 42 (13.04) | | |
| | 21 | 58, 25 (13.20)[d] | | 111, 9 (12.7)[d] |
| | | 55 (13.0) | | 11 (12.4) |
| | 27 | 111 (13.2) | | 19 (12.4) |
| bis(acridine) pyrazole–$d(AT)_5 \cdot d(AT)_5$ (BAPY) | 20 | 50, 9 (13.25)[d] | | 8 (12.48) |
| | | | | 8 (11.9) |
| | | | | 8 (11.7) |
| | 27 | 62 (13.2) | | 40 (12.38) |
| | | | | 16 (11.9) |

[a] Values reported for $R_1$ have an error of ~10%; numbers in parentheses are chemical shifts in ppm. [b] Only inner base pair values are tabulated for comparison. See Table II. [c] Peaks have coalesced, but different rates of recovery are discernible. [d] Biexponential decay observed, and two rates reported. Main component is underlined. [e] See Table II.

relaxation measurements, whereas most of the other resonances in the drug complex have relaxation rates that are comparable to the most stable resonances observed in free $d(AT)_5 \cdot d(AT)_5$ (see Table III). By 32.5 °C, resonances at 12.7 and 12.2 ppm exchange at a rate of 80–90 $s^{-1}$ (data not shown), and it is difficult to ascertain whether we are monitoring a "coalesced" peak or only drug-shifted peaks that have survived the higher temperature conditions.

*Bis(acridine) Spermine–$d(AT)_5 \cdot d(AT)_5$ Complex.* The spin–lattice and spin–spin relaxation behavior of the $d(AT)_5 \cdot d(AT)_5$–BAS complex at 7 °C is shown in Figure 7 and tabulated on Table II. The most upfield shifted resonance at 12.4 ppm and the unshifted resonance at 13.3 ppm have relaxation rates comparable to the inner base pairs (13.26 ppm) in free $d(AT)_5 \cdot d(AT)_5$ (~4 $s^{-1}$). Some nonexponentiality, apparent at early times in the decay of the 12.40 ppm resonance, could be due to contributions from the faster recovery of the partially overlapping resonance centered at 12.7 ppm. The rapidly recovering resonance at 13.04 ppm ($R_1$ ~ 42 ± 5 $s^{-1}$) is tentatively assigned to the terminal base pair because of its chemical shift, its relaxation behavior, and its temperature dependence. The drug-shifted imino resonances at ~12.7 ppm have the fastest spin–lattice and spin–spin recovery rates, suggesting that even at 7 °C these imino protons are *more* susceptible to exchange with solvent than are other imino protons in the complex.

The spin–lattice relaxation behavior of the bis(acridine) spermine–$d(AT)_5 \cdot d(AT)_5$ complex at 21 and 27 °C is shown in Figure 8. At these temperatures, $R_1$ is faster because exchange with solvent dominates relaxation and comparisons between the free $d(AT)_5 \cdot d(AT)_5$ and the bis(acridine)–DNA complexes are more appropriate than at 6 °C. At 21 °C, the

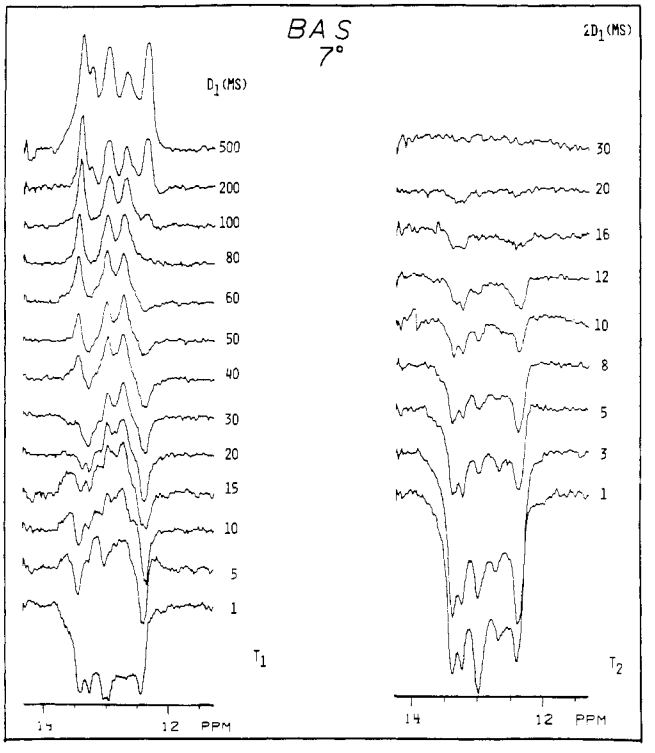



FIGURE 7: Experimental spectra (360 MHz) obtained from longitudinal and transverse relaxation measurements on a complex of bis(acridine) spermine and $d(AT)_5 \cdot d(AT)_5$ measured at 7 °C.

drug-shifted resonances at ~12.4 ppm are *kinetically* more stable with respect to exchange than are the unshifted resonances at ~13.2 ppm; the ~12.7 ppm resonances continue

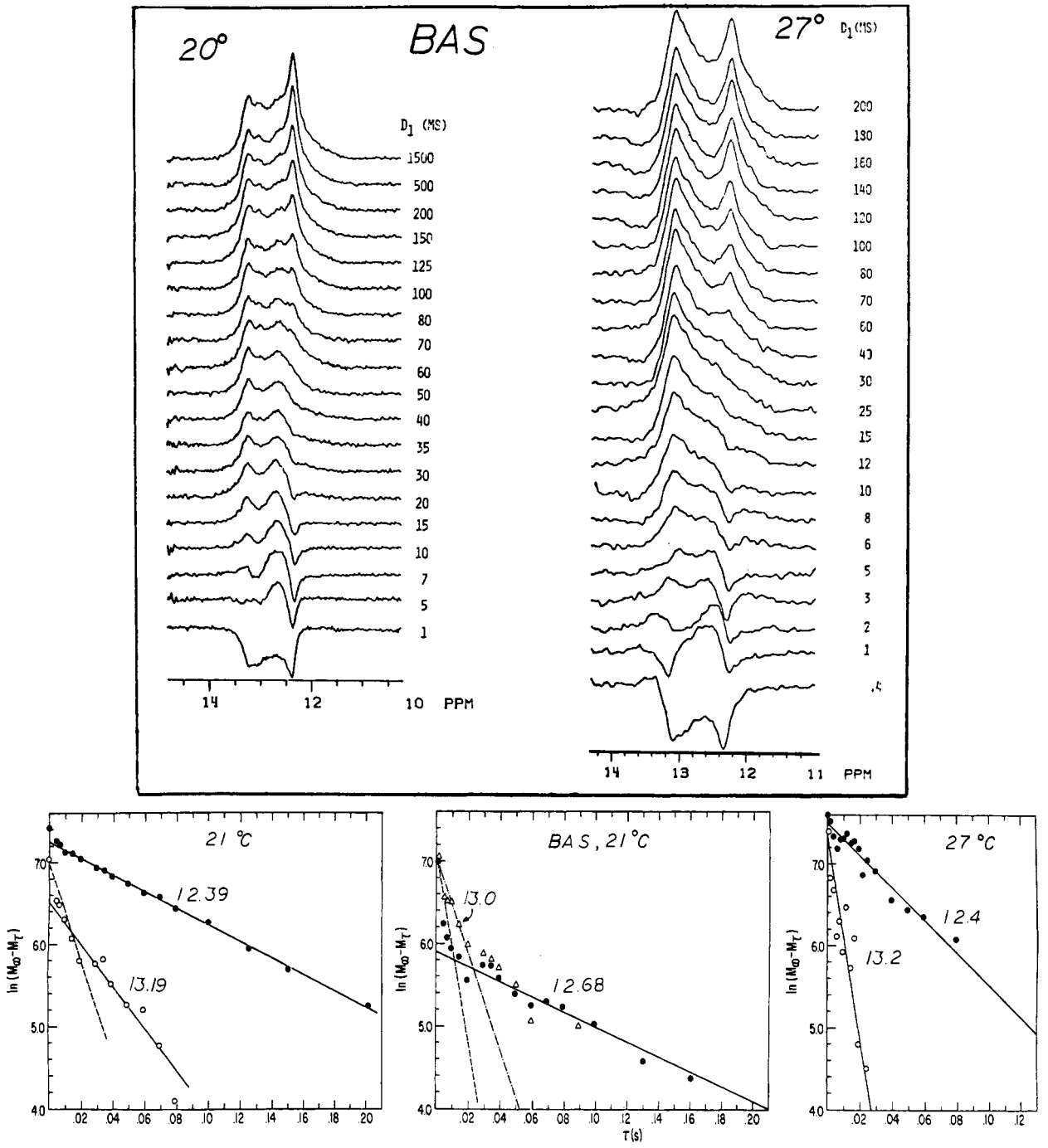



FIGURE 8: (Top) Spectra obtained from longitudinal relaxation measurements at 20 and 27 °C of a bis(acridine) spermine–$d(AT)_5$·$d(AT)_5$ complex. (Bottom) Semilog plots showing the longitudinal relaxation drug-shifted (~12.4 and 12.7 ppm) and unshifted (~13.2 and 13.0 ppm) imino resonances in a bis(acridine) spermine–$d(AT)_5$·$d(AT)_5$ complex at 21 and 27 °C.

to be the fastest to recover in the complex. By 27 °C, only two groups of resonances are clearly discernible: unshifted imino resonances arising from base pairs with no intercalated drug neighbor and the resonances most shifted upfield by an adjacent drug chromophore. At 27 °C the relaxation of the drug-shifted resonances in the bis(acridine) spermine–DNA complex (19 $s^{-1}$) is ~4.5 times slower than the unperturbed resonances in the same molecule (~111 $s^{-1}$) or the imino resonances of free $d(AT)_5$·$d(AT)_5$ at the same temperature (see Table III).

*Bis(acridine) Pyrazole (BAPY).* To explore the effect the character of the linker chain has on the imino proton exchange rates, we also studied the relaxation behavior of 1:1 complexes of $d(AT)_5$·$d(AT)_5$ with BAPY, a bisintercalator with a less flexible interconnecting linker chain. At 20 °C, resonances arising from the unperturbed and the drug-shifted imino protons are still well resolved (Figure 9). The drug-shifted resonances at 11.7, 11.9, and 12.5 ppm all have slow spin–lattice relaxation rates (~8 $s^{-1}$) whereas the unperturbed imino resonances at ~13.25 ppm exhibit a more complex, nonexponential spin–lattice recovery (Figure 9 and Table III). The initial "fast" decay, which persists well beyond the first 10 ms of observation (and cannot be neglected), has a rate of >60 $s^{-1}$ (100 $s^{-1}$). At longer times, a slower component (~9 $s^{-1}$) is also observed for all resonances. At least two interpretations of these data are possible: (1) The fast component at 20 °C might be due to faster relaxing aggregates, but if this were correct, all resonances should have a fast decay component. (2) Binding of the BAPY to the $d(AT)_5$·$d(AT)_5$ has stabilized the decamer, making it possible to observe imino resonances

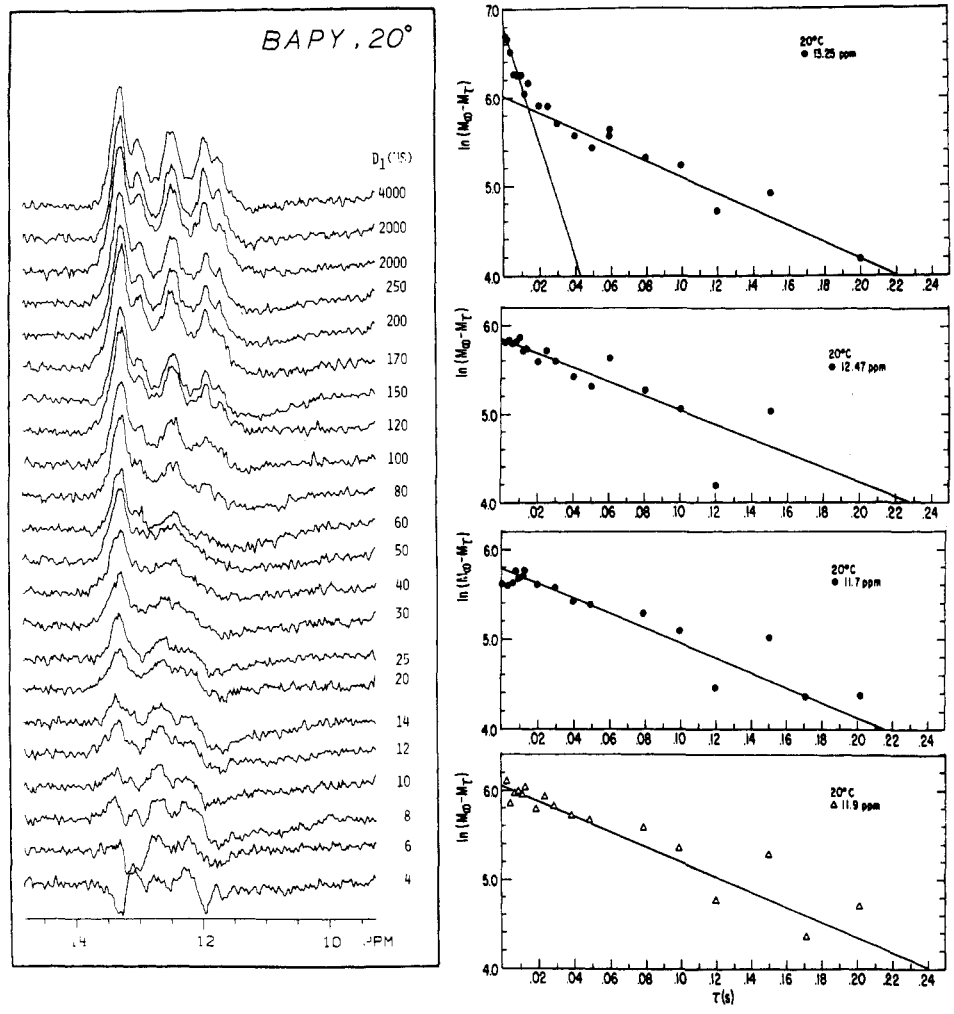



FIGURE 9: Experimental spectra obtained from longitudinal relaxation measurements at 20 °C on a bis(acridine) pyrazole–d(AT)$_5$·d(AT)$_5$ complex (left) and semilog plots (right) of the longitudinal relaxation at 20 °C of individual resonances.

arising from base pairs distant from the binding site and heretofore unmeasurable at 20 °C because of fast exchange.

At 27 °C, the relaxation rates for the different drug-shifted imino resonances are no longer the same. The less shifted resonances at ~12.4 pm have a relaxation of ~39 s$^{-1}$, while the highly shifted resonances at 11.9 ppm have a rate of ~16 s$^{-1}$ (Table III). The slow component in the relaxation of the 12.4 ppm resonance is attributed to contributions from overlapping, slower relaxing resonances. Since differences in relaxation rates at this temperature are due to differences in proton exchange with solvent, these results suggest that the most shifted resonances are also the most protected against exchange. We note that the relaxation rate of the unshifted resonances, 62 s$^{-1}$, is comparable to that observed for the most stable base pairs in free d(AT)$_5$·d(AT)$_5$ (~80 s$^{-1}$) at the same temperature (see Table III).

## Discussion

In this study we have measured the spectra and spin–lattice relaxation rates of the drug–DNA complexes and free d(AT)$_5$·d(AT)$_5$ at various temperatures. The relaxation measurements ($R_1$ and $R_2$) provide direct information on the kinetic stability of base pairs with respect to breathing (manifested through proton exchange with water protons) and the effects the different drugs have on the base pair opening rates. This information, when combined with measurement of the spectra at different temperatures, also provides information regarding the lifetime of the intercalated drugs at specific binding sites. We begin by considering the behavior of free d(AT)$_5$·d(AT)$_5$, and then the behavior of complexes of 9AA and the four bis(acridine) with d(AT)$_5$·d(AT)$_5$ is analyzed.

*Free d(AT)$_5$·d(AT)$_5$.* At 6 °C, resonances from the imino protons of all 10 base pairs are present. At low temperature the opening rates are slow for the interior base pairs (at 6 °C) and most of the relaxation can be attributed to dipolar interactions (Table II). Even at 6 °C the terminal base pair protons (13.03 ppm) exchange rapidly with water (manifested by the fact that $R_1 = R_2$). At higher temperature (21 and 27 °C), however, the integrated intensity decreases because proton exchange with solvent is fast and the spin–lattice relaxation rates (Table III) provide a direct measure of the base pair opening rates (and the corresponding rates of exchange with water protons). On the basis of their relaxation behavior and the order in which they broaden with increasing temperature, we have suggested assignments for the five low-field resonances observed in the 6 °C spectrum of d(AT)$_5$·d(AT)$_5$, and these are summarized in Figure 1. The spectra and relaxation data for free d(AT)$_5$·d(AT)$_5$ provide a basis for comparison with and interpretation of the properties of the drug complexes with d(AT)$_5$·d(AT)$_5$.

*9AA–d(AT)$_5$·d(AT)$_5$.* At low temperatures, the 2.2:1 9AA complex with d(AT)$_5$·d(AT)$_5$ exhibits an upfield shifted resonance at 12.5 ppm with intensity corresponding to about 50% of the total integrated intensity (see Figure 2). As discussed in the preceding paper (Assa-Munt et al., 1985), this is consistent with 100% intercalation of all the 9AA added. When

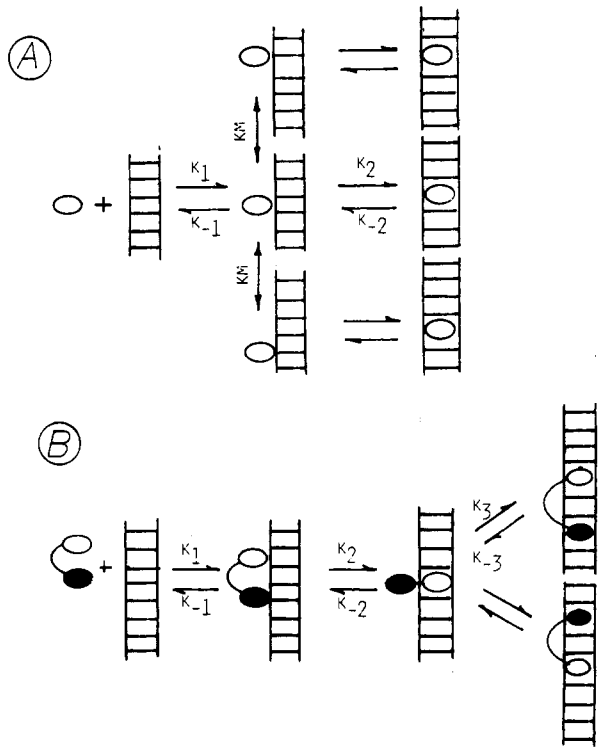



FIGURE 10: Kinetic schemes depicting the binding of monointercalating (A) and bisintercalating (B) drugs to DNA. For the bisintercalator, the two chromophores have been differently marked, although in the present case they are identical.

the sample is heated to 17 °C, there is a slight overall loss of intensity, and the two groups of resonances originally present in the 6 °C spectrum coalesce into a single broad peak. Note that the main resonance in the unperturbed DNA spectrum starts out at ~13.3 ppm at 6 °C. Therefore, the coalesced peak observed at 12.6 ppm at 17 °C is midway between peaks observed in free DNA and the highly shifted resonances (12.3 ppm) measured at 6 °C. One possible explanation for this behavior might be that, at the higher temperature, the imino protons are beginning to rapidly exchange with water with a corresponding loss of resolution. This possibility is eliminated, however, by the observation that the major collection of resonances (located around 12.6 ppm) has relaxation rates (~24–30 $s^{-1}$) comparable to those of the most slowly exchanging resonances in free $d(AT)_5 \cdot d(AT)_5$ at 21 °C. Moreover, an exchange rate of 30 $s^{-1}$ would contribute only ~10 Hz to the line widths which start out at ~100 Hz, and therefore, the observed coalescence cannot be due to accelerated exchange. Instead, the coalescence must be attributed to rapid exchange of the 9AA molecules between different possible intercalation sites.

To clarify the relation between the NMR and other types of experiments that have been carried out, consider the kinetic scheme depicted in Figure 10A. According to this scheme, the lifetime of an intercalated drug in a specific binding site is $t = (k_{-2})^{-1}$. For the simple case of exchange between two equally populated sites with resonance frequencies $\nu_A$ and $\nu_B$, respectively, the two peaks coalesce when the jump rate $k_{-2} = (\pi/2^{1/2})(\nu_A - \nu_B)$ (Sutherland, 1971 and references therein). For 9AA–$d(AT)_5 \cdot d(AT)_5$ complex at low temperature $\nu_A$ = 13.0 ppm and $\nu_B$ = 12.6 ppm so that coalescence, which occurs at about 17 °C, indicates that the $k \sim 270$ $s^{-1}$. Alternatively, we can obtain an upper limit to the value of the jump rate by assuming that all of the increased line width of the drug-shifted resonance ($\Delta\nu$) can be attributed to increased rate of exchange between binding sites. From the spectra shown in Figure 8, we estimate that the drug-shifted resonance broadens by about 100 Hz on heating from 6 to 15 °C, indicating that the jump rate at 15 °C is $k_{-2} < \pi\Delta\nu \sim 300$ $s^{-1}$. Thus, the lifetime of the 9AA molecules in a particular binding site is estimated to be ~3 ms at 17 °C. We note that the jump rate obtained here for 9AA is similar to the values previously deduced from NMR measurements on ethidium complexes of DNA (Feigon et al., 1982).

It is important to note that the jump rates obtained by these NMR measurements will not necessarily correspond to *off*-rate constants obtained by other types of measurements (e.g., rate of intramolecular transfer of drugs *between* DNA molecules, sequestering experiments, *T* jump). In the limiting case where dissociation of nonintercalated drug from the DNA is slow compared with the rate of intercalation and disintercalation (i.e., $k_2 > k_{-2} > k_{-1}$) stopped-flow and other types of kinetic experiments would measure $k_{-1}$, and consequently, the rate $k_{-2}$ measured in our NMR experiments could be quite different. In the other limit, where disintercalation is the slow step in the complete dissociation of the drug from the DNA (i.e. $k_{-1} > k_{-2}$), both the NMR and the other kinetic experiments would yield the same rate constant for dissociation from the DNA, and the rate of bisintercalation would be nearly the same. In intermediate cases, more complex kinetics would be observed.

Although 9AA stabilizes DNA duplexes against thermal denaturation (Patel, 1977), this does not necessarily imply that it kinetically stabilizes the base pairs at the binding site. In fact, the intercalation process could actually facilitate the exchange of the base pair protons. Experimentally we find that the imino proton relaxation rates are similar in free $d(AT)_5 \cdot d(AT)_5$ and the 9AA complex with $d(AT)_5 \cdot d(AT)_5$ at 19–21 °C, indicating that the intercalation of 9AA has little effect on the exchange behavior of the adjacent AT base pairs. Moreover, since the lifetime of the drug in a specific site is short (<4 ms), it may be further concluded that the 9AA intercalation and dissociation processes do not measurably affect the exchange rates.

The rapid migration of the 9AA at 19 °C suggests a possible explanation for the observation that addition of drug to $d(AT)_5 \cdot d(AT)_5$ at 6 °C not only generates a collection of highly shifted resonances but also causes some shift of the remaining less perturbed resonances. If the chromophore has a preference for one type of binding site (ApT or TpA; probably TpA) (Reinhardt & Krugh, 1978) and makes short-lived "excursions" to the weaker binding site (ApT) for short periods of time, all resonances in the $d(AT)_5 \cdot d(AT)_5$ could be affected.

*Dynamic Behavior of Bisintercalators.* In order to discuss the kinetic aspects of the interaction of bisintercalating drugs with DNA, consider the simplified kinetic scheme shown in Figure 10B. On comparing this scheme with the one appropriate for a monointercalator, we see that the off-rate constant, $k_{-1}$ and $k_2$, $k_{-2}$ have the same significance in both schemes, but a second pair of constants, $k_3$ and $k_{-3}$, have to be added to account for the intercalation of the second chromophore. With the bisintercalating drugs, the NMR is sensitive to $k_{-3}$ (and perhaps to $k_{-2}$ provided $k_{-2} > k_{-3}$). Since the low-temperature experiments (Assa-Munt et al., 1985) demonstrate that the drugs chosen for this study bisintercalate, we know that $k_1 > k_{-1}$ and $k_2 > k_{-2}$, and that $k_3 > k_{-3}$. Therefore, migration of the drug along the DNA by a creeping mechanism will be limited by the rate $k_{-3}$. Since the fraction of drug bound simply to the outside of the DNA is judged to be small, creeping may provide a relatively fast mechanism for transport along the DNA. Having presented a simple kinetic scheme

to describe the binding of the bisintercalators, we now consider the effect of temperature on the low-field spectra and the spin–lattice relaxation behavior of each of the bis(acridine)–$d(AT)_5$·$d(AT)_5$ complexes.

*$C_8$*. The $C_8$–$d(AT)_5$·$d(AT)_5$ complex is in the slow exchange limit at 6 °C since resolved upfield shifted resonances are observed (Figure 3). However, by ~20 °C, the drug is in rapid exchange among the different binding sites as evidenced by a coalescence of resonances in the low-field spectrum. The possibility that this coalescence arises from rapid proton exchange with the solvent is eliminated by the observation that the spin–lattice rate for these resonances (60–70 $s^{-1}$) could account for only a 10–15-Hz increase in line widths over those observed at low temperatures (see Table III). At 21 °C we, therefore, estimate that the jump rate is on the order of 400–500 $s^{-1}$. The fact that the spin–lattice relaxation rates measured at 21 °C are twice as large as those measured for free $d(AT)_5$·$d(AT)_5$ at the same temperature suggests there is some slight kinetic destabilization of base pairs upon drug binding.

*$C_{10}$*. The complex of $C_{10}$ with $d(AT)_5$·$d(AT)_5$ appears to be kinetically more stable than the $C_8$ complex in that it exhibits resolved peaks up to 20 °C, at which point broadening of most resonances in the low-field spectrum is noticed in free DNA (see Figure 3). Even at 29 °C, drug-shifted resonances are still present in the spectrum although the unshifted d-$(AT)_5$·$d(AT)_5$ resonances have been substantially broadened. Thus, in contrast with the behavior of the $C_8$–$d(AT)_5$·$d(AT)_5$ complex, we do not see evidence for rapid migration of the drug at temperatures up to 30 °C, and this suggests that the longer linker chain has suppressed the rate of migration along the DNA. Judging from the extent of broadening observed in the 29 °C spectrum, the rate of migration of the drug among sites on the DNA must be >100–200 $s^{-1}$. The relaxation measurements indicate that binding of the drug to $d(AT)_5$·$d(AT)_5$ has two different effects on the A·T base pairs at 6 °C (see Table III). The drug-shifted resonance at 12.26 ppm contains a rapidly decaying component (rate constant ~50 $s^{-1}$), indicating that binding has kinetically destabilized some base pairs at the binding site. The other drug-shifted resonances relax only slightly faster than those observed with free d-$(AT)_5$·$d(AT)_5$ (due perhaps to the slower tumbling of the drug complex or possibly some aggregation). The unperturbed resonances have similar values in the free DNA and in the complex. At elevated temperatures (20 and 32 °C) the relaxation rates of all resonances in the spectra of the complex are comparable to those observed with free $d(AT)_5$·$d(AT)_5$, and this suggests that the drug binding has had little effect on the kinetic stability of these remaining resonances.

The behavior of the $C_{10}$ complex is to be contrasted with that observed for complexes with bis(acridine) pyrazole and bis(acridine) spermine.

*Bis(acridine) Pyrazole (BAPY)*. Bis(acridine) pyrazole produces a striking stabilization of drug-shifted resonances relative to unshifted ones. At the lower temperatures (11 and 16 °C) three groups of resonances are still visible for the 1:1 BAPY–$d(AT)_5$·$d(AT)_5$ complex (Figure 3). Resonances at ~13.2 ppm are attributed to unshifted resonances, arising from unperturbed imino protons. The most highly shifted resonances, those at ~11.9 and 11.7 ppm, are attributed to base pairs "sandwiched" between the two chromophores (Figure 3), and the less shifted resonances at ~12.4 ppm are ascribed to the imino protons from base pairs with a chromophore on one side and a normal base pair on the other.

At 20 °C, the drug-shifted resonances exhibit comparable (~8 $s^{-1}$) relaxation rates (Table III). The unshifted resonances exhibit markedly biexponential decay with rates of ~9 and ~50 $s^{-1}$. We suggest that the faster relaxation rate arises from imino protons distant from the binding site which are now observable because their exchange rate with solvent has been slowed down. At 27 °C the most shifted resonances relax at a rate at least 2 times slower than the less shifted resonances. The unshifted resonances, on the other hand, exhibit fast relaxation at a rate (~60 $s^{-1}$) comparable to that observed for free $d(AT)_5$·$d(AT)_5$ and for unshifted $d(AT)_5$·$d(AT)_5$ resonances in other drug complexes (~80 $s^{-1}$) at this temperature. The differential stability of the drug-shifted resonances is also clearly evident in the 37 °C spectrum (Figure 3) where broadened drug-shifted resonances are still present, but the unshifted resonances have nearly disappeared. These observations demonstrate that bisintercalation of BAPY has kinetically stabilized the base pairs sandwiched between the chromophores and those adjacent to the sandwich. Line-width analysis of the 300-MHz spectrum of the BAPY–$d(AT)_5$·d-$(AT)_5$ complex indicates a lifetime for the chromophores in their binding sites of >20 ms at 16 °C. Such behavior must be contrasted with the one observed for $C_{10}$, a bis(acridine) with a linker chain of comparable length (13.8 Å). Obviously, one of the factors contributing to longer residence time of BAPY must be the rigid linking chain, which hinders "crawling" of the drug along the DNA (Le Pecq et al., 1975).

*Bis(acridine) Spermine (BAS)*. When bis(acridine) spermine, a bisintercalator with a charged linker chain, binds to $d(AT)_5$·$d(AT)_5$, the shifted imino resonances exhibit very different degrees of protection by the drug toward exchange with solvent. The spin–lattice relaxation rates obtained for a 1:1 complex of bis(acridine) spermine–$d(AT)_5$·$d(AT)_5$ at 6, 20, and 27 °C (summarized in Tables II and III) show that the most shifted resonance in this complex (12.4 ppm) exhibits a much slower (~5-fold) relaxation rate than the unshifted imino resonances, throughout the temperature range studied. The behavior of the less shifted resonance, at 12.7 ppm, is anomalous, however, in that it exhibits the fastest $R_1$ and $R_2$ rates in the complex at low temperature. These extremely fast rates can be accounted for only if the imino protons which resonate at 12.7 ppm are exposed to solvent and thus have already a facilitated exchange path with water protons. The possibility that this faster relaxation arises from formation of aggregates is dismissed in this case, since *all* of the resonances would exhibit faster relaxation rates, whereas the 13.26 and 12.4 ppm resonances exhibit relaxation rates (~4 $s^{-1}$) comparable to those of free $d(AT)_5$·$d(AT)_5$ at that temperature. At 21 °C (see Figure 8) the 12.4 ppm resonances continue to have very slow relaxation rates (11 $s^{-1}$), but for the unshifted imino resonances at ~13.2 ppm, two relaxation rates are discernible, a faster one (~58 $s^{-1}$) and a slower one (~25 $s^{-1}$), which is comparable to that of free $d(AT)_5$·$d(AT)_5$ at this temperature. Whether the faster rate of 58 $s^{-1}$ corresponds to an imino proton which originally was exchanging too fast to be observable in free $d(AT)_5$·$d(AT)_5$, and is now slowed down in the drug complex, or represents destabilization of imino protons not directly at the drug binding site is unclear. In this regard, we note that Pardi et al. (1983) observed a *destabilization* of imino protons due to the addition of actinomycin to a dodecamer $d(CGCGAATTCGCG)_2$ which already contained a bound molecule of netropsin (a charged outside binder). Thus, there may be unfavorable interactions present when both ionic and intercalative modes of binding must coexist.

At 27 °C, only two groups of resonances (at 13.2 and ~12.3 ppm) are still clearly discernible, and again (Figure 8), the most shifted resonances (exchange rate of ~20 $s^{-1}$) are much more stabilized (4–5×) than the unshifted ones, those of free $d(AT)_5$·$d(AT)_5$ at this temperature. By 37 °C the unshifted resonances have disappeared, but the 12.4 ppm resonances remain relatively well resolved (Figure 3). This indicates that, up to this temperature, migration of the drug among different binding sites is slow on the NMR time scale (i.e., jump rates less than 100 $s^{-1}$).

One final point regarding the bis(acridine) spermine complex concerns the appearance of a new, broad resonance centered around 11.5 ppm in the high-temperature spectra. The unusual position and stability of these new resonances have no obvious origin. One possibility is that they represent unusual base pairing arrangements that have been stabilized by the bis(acridine). Alternatively, they may arise from new hydrogen bonding between the bis(acridine) and the d-$(AT)_5$·$d(AT)_5$. The fact that such resonances are absent at low temperatures where all bases are present in Watson–Crick pairs is puzzling, however.

The correlation between the physicochemical parameters studied and the biological activity of these drugs may be illustrated by the finding that in biological tests performed at the University of Auckland School of Medicine, BAPY is active in inhibiting tumor cell growth at concentrations 400 times smaller than those needed for other bis(acridine) compounds [e.g., $Amd_8$; see Assa-Munt et al. (1985) and shows in vivo antitumor activity (Denny et al., 1984a). The higher activity of this compound may be attributed not only to higher binding constants but also to slower mobility of this drug on the DNA, brought about by the reduced flexibility of the linking chain. The suggestion by Le Pecq et al. (1975) that a trisintercalator might stay bound to the DNA even longer times must be explored, and a recent report (Atwell et al., 1983) announces the successful synthesis of a tris(acridine) trisintercalator.

## Conclusions

It is evident from our studies of the effect of temperature on the spectra and the relaxation rates of the bis(acridine) complexes with $d(AT)_5$·$d(AT)_5$ that the nature of the linker chain plays an important role in determining the stability of the imino protons at the binding site. $C_8$ and $C_{10}$, with flexible linker chains, offer little protection toward exchange. Bis(acridine) spermine, which is anchored to the DNA helix by a charged linker, presents mixed results, with some of the drug-shifted imino protons actually exchanging faster with solvent, while other drug-shifted imino protons in the same complex are highly protected.

The results of the effect of temperature on complexes of $d(AT)_5$·$d(AT)_5$ and bis(acridine) pyrazole point to a remarkable stabilization of the DNA imino protons by this drug. Le Pecq et al. (1975) postulated that slowing down of the "creeping" motion of the drug on the DNA helix may be advantageous with respect to enhancing the chemotherapeutic effects of these drugs. Now that parameters such as lifetime of the drug in the binding site and protection of base pairs toward exchange with solvent can be measured, these may be useful in the search of active drugs, which may efficiently compete with the binding of enzymes (e.g., RNA polymerase), be more sequence specific, and perhaps display higher antitumor selectivity.

## Acknowledgments

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**Registry No.** 9AA, 90-45-9; BAPY, 94731-75-6; BAS, 58478-35-6; $C_8$, 57780-57-1; $C_{10}$, 21988-21-6; $d(AT)_5$, 85240-23-9.

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