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Proton Nuclear Magnetic Resonance Investigations of the Nucleation and Propagation Reactions Associated with the Helix–Coil Transition of d-ApTpGpCpApT in H_2O Solution†

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ABSTRACT: The chemical shifts and line widths of the Watson–Crick ring NH resonances of the self-complementary duplex of d-ApTpGpCpApT have been monitored in the presence of 0.1 *M* phosphate at neutral pH in aqueous solution. While the resonance positions of the terminal and internal AT base pairs shift upfield and broaden as average resonances with increasing temperature (helix and coil exchange several times prior to exchange with water from the coil form), those of the central GC base pairs broaden in the absence of upfield shifts (exchange with water occurs each time helix converts to coil). The line-width changes at the AT base pairs monitor the lifetime of the coil state at these positions prior to exchange with water while the line-width changes at the GC base pairs monitor the lifetime of the helix prior to dissociation to strands. This permits the separation of the propagation reaction at the AT base pairs from the nucleation reaction at the GC base pairs during helix formation. The experimental data have been quantitatively analyzed to yield (at 20°) a nucleation formation rate of $\sim 10^3$ sec^{-1} for the GC base pairs (bimolecular rate constant of $\sim 6 \times 10^6$ l. mol^{-1} sec^{-1}) and a dissociation rate of 6×10^2 sec^{-1} at these same base pairs (unimolecular dissociation to strands). The unimolecular propagation reactions at the internal and terminal base pairs are associated with reaction rates $\gg 10^4$ sec^{-1}. These values are consistent with a slow formation of a stable nucleus at the GC base pairs followed by a rapid propagation reaction at the AT base pairs. The line width of the $(GC)_{central}$ base pairs in the presence of phosphate is a direct measure of the lifetime of the total helix and yields an activation energy of 45 kcal for helix to coil conversion measured over a narrow temperature range. The exchange from the coil form with water is catalyzed by 0.1 *M* phosphate with a rate constant $k_{HPO_4^{2-}} = 0.2 \times 10^6$ l. mol^{-1} sec^{-1}.

Equilibrium and temperature jump optical experiments on short, well-defined RNA and DNA helices have recently provided considerable insight into the stability of nucleic acid double helices and into the mechanism of helix formation (for review see Riesner and Römer, 1973; Bloomfield et al., 1974). Such data have been analyzed in terms of a model which describes the generation of a nucleic acid double helix from its component single strands by a concentration dependent formation of the first few base pair(s) (the nucleus) followed by a fast formation of the subsequent base pairs (propagation) (Pörschke and Eigen, 1971; Craig et al., 1971).

Nuclear magnetic resonance is capable under conditions of high spectral resolution of unravelling many of the finer details of the dynamics and mechanisms involved in the helix–coil transition of nucleic acids. In the preceding papers of this series, proton nuclear magnetic resonance (NMR) spectroscopy was utilized to demonstrate that the double helix formed by the hexanucleotide d-ApTpGpCpApT opens in a sequential manner from its ends (Patel, 1974), and the thermodynamic parameters associated with the fraying of the individual base pairs were determined and interpreted (Patel and Hilbers, 1975). These studies are

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extended below to describe the separation and characterization of the nucleation reaction from the propagation reaction in d-ApTpGpCpApT and in addition provide estimates of the kinetic parameters associated with the different steps.

The experimental conditions have been described in the previous paper (Patel and Hilbers, 1975).

Results

I. Spectra. A. TEMPERATURE DEPENDENCE. It is instructive to compare the line-width changes with temperature in representative spectra of d-ApTpGpCpApT in the absence and presence of 0.1 *M* phosphate (Figure 1). The comparison demonstrates that the $(AT)_{terminal}$ and $(TA)_{internal}$ base pairs exhibit much greater ring NH line widths in the presence of 0.1 *M* phosphate at a given temperature. The $(GC)_{central}$ resonance exhibits the same line width in the presence and absence of phosphate in the range 10–26° (conditions under which aggregation is minimum at high salt concentrations, i.e., 0.3 *M* NaCl–0.1 *M* phosphate).

The chemical shifts and line widths in the presence of phosphate are plotted in Figure 2A as a function of temperature.

The upfield chemical shift vs. line width for the ring NH of the GC central base pair as a function of solvent composition is plotted in Figure 2B.

B. CONCENTRATION DEPENDENCE. Under the solution conditions described above, the line width of the $(GC)_{central}$ base pair first decreases (−5 to +10°) and then increases (10–26°) (see Figure 2A). In order to get some insight into the line-width dependence at low temperature, spectra were measured at two different hexanucleotide concentrations. The line widths of the G-N_1H resonance in 0.3 *M* NaCl–0.1 *M* phosphate–H_2O (pH 7) at 0° are ~40 and ~25 Hz for 25 and 5 mg/ml single strand concentration, respectively (Figure 3). This and the temperature dependence of the line widths of the 25 mg/ml single strand concentration from −5 to 10° suggest that aggregation may play a role in this temperature range.

In addition, there appears to be a small dependence of the chemical shift of the NH resonance of the $(GC)_{central}$ base pair on the concentration of the hexanucleotide. The ring NH resonance shifts 0.05 ppm upfield at the higher hexanucleotide concentration (Figure 3).

II. Analysis. A. AT BASE PAIRS. The temperature dependence of the chemical shifts of the ring NH resonances of the $(AT)_{terminal}$ and $(TA)_{internal}$ base pairs for d-ApTpGpCpApT in 0.3 *M* NaCl–0.1 *M* phosphate–H_2O (pH 7) presented in Figure 2A is accompanied by line broadening. Since the exchange process involves rapid interconversion between helix and coil several times prior to exchange with water (case A), the excess broadening associated with the upfield shifts is given by $f_C/\pi\tau_{CW}$ (Crothers et al., 1975).

The temperature dependent τ_{CW}^{-1} values of the terminal and internal base pairs of d-ApTpGpCpApT at low ionic strength and in the presence of phosphate (0.3 *M* NaCl–0.1 *M* phosphate) in aqueous solution at neutral pH are plotted against inverse absolute temperature in Figure 4.

B. GC BASE PAIRS. The phosphate experiments were initiated under the assumption that addition of enough phosphate would result in a large increase of the rate of proton transfer from the ring NH in the coil state to solvent water. The exchange situation could be switched from case A (helix and coil interchange many times before exchange of the ring N proton with water takes place) to case B (exchange with water occurs virtually every time the helix is opened).

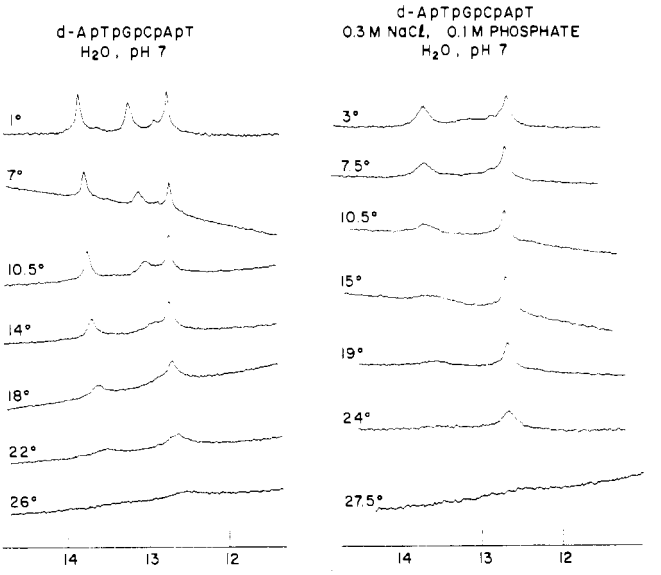

FIGURE 1: A comparison of the ring NH resonances of d-ApTpGpCpApT between 0 and 30° recorded in H_2O, low ionic strength, pH 7, and in 0.3 *M* NaCl–0.1 *M* phosphate (pH 7).

For the latter situation, case B, solution of the three site exchange equation (Crothers et al., 1975) predicts that the resonance will broaden without shifting its position from that of the fully helical state, ω_H, and that the excess line width is determined by τ_H^{-1}, the lifetime of the proton in the helix.

The observed chemical shift changes that accompany the line broadening of the ring NH of the $(GC)_{central}$ base pair with increasing temperature as a function of solvent are plotted in Figure 2B. In the presence of phosphate (0.3 *M* NaCl–0.1 *M* phosphate–H_2O (pH 7)), the ring NH of central base pair broadens out completely with increasing temperature in the absence of any significant upfield shift from the fully helical chemical shift value. This means that in the presence of phosphate, case B applies to the NH resonance of the $(GC)_{central}$ base pairs, while case A still applies, as presented in section IIA, to the NH resonances of the $(AT)_{terminal}$ and $(TA)_{internal}$ base pairs. As will be discussed, this permits *the separation of the nucleation from the propagation reaction during helix formation.*

The helix lifetime, τ_H^{-1}, as evaluated from the excess line width of the GC resonance in the presence of phosphate, is plotted as a function of inverse absolute temperature in Figure 5.

Discussion

I. Catalysis by Phosphate. Comparison of the values of the transition probability for exchange from the coil form to water $(\tau_{CW})^{-1}$, so obtained in H_2O, low ionic strength, pH 7, and in 0.3 *M* NaCl–0.1 *M* phosphate–H_2O (pH 7) indicate catalysis by phosphate. Since the total phosphate concentration is 0.1 *M*, the $[HPO_4^{2-}]$ concentration at pH 7 is 0.045 *M*, so that $k_{HPO_4^{2-}} = 0.2 \times 10^6$ sec^{-1} mol^{-1}. Surprisingly, this is a much lower value than would be expected on the basis of the p*K* difference between uracyl and the HPO_4^{2-} ion (Eigen, 1963). If this effect is due to the double negative charge of the phosphate ion, it may be worthwhile to undertake future experiments in neutral buffer.

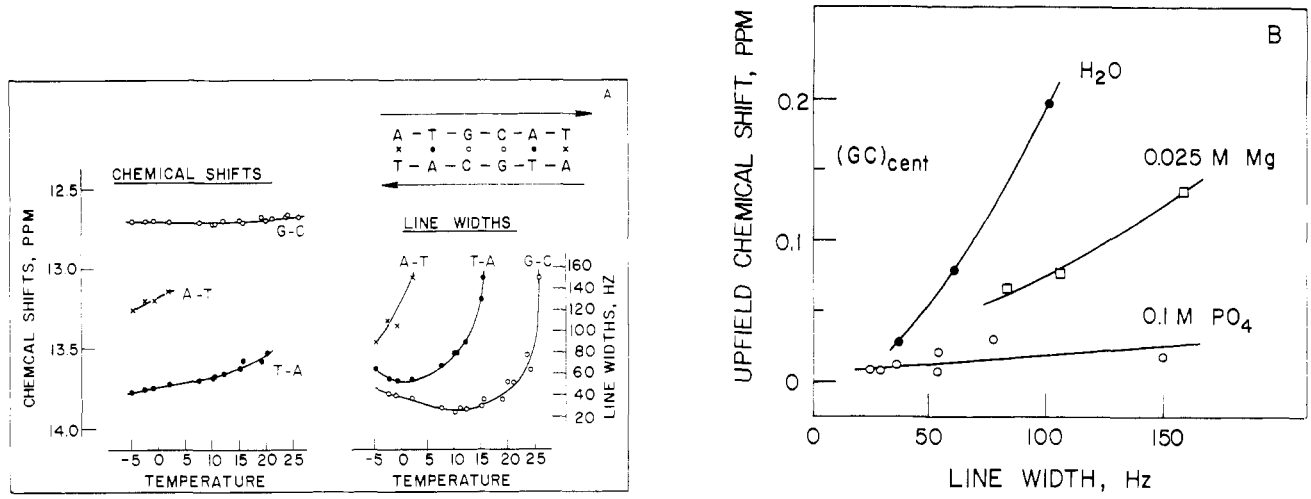

FIGURE 2: (A) Plots of chemical shifts and line widths of d-ApTpGpCpApT as a function of temperature in 0.3 *M* NaCl-0.1 *M* phosphate-H_2O (pH 7). (B) A plot of the upfield chemical shift vs. change in line width of the G-N_1H resonances of the GC central base pairs as a function of solvent.

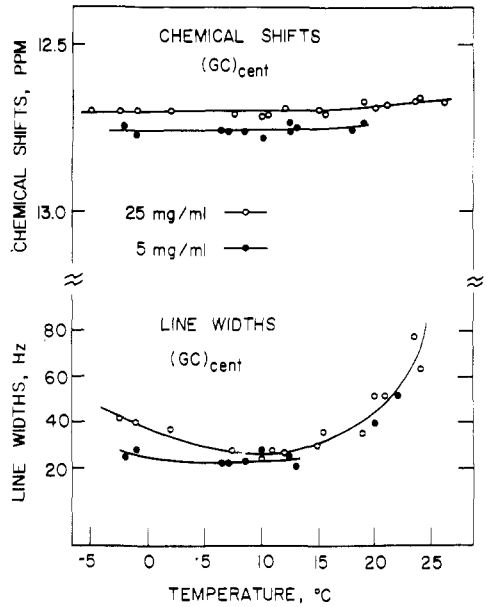

FIGURE 3: Comparison of the chemical shifts and line widths of the ring NH of the GC resonance in d-ApTpGpCpApT in 0.3 *M* NaCl-0.1 *M* phosphate-H_2O (pH 7) at single strand concentrations of 25 mg/ml (14 m*M*) and 5 mg/ml (2.8 m*M*).

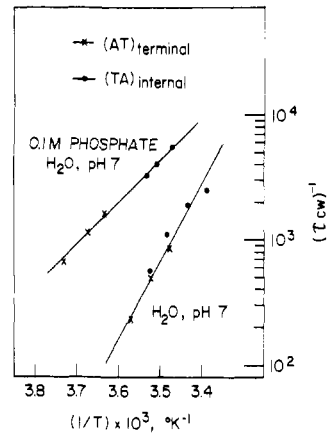

FIGURE 4: Plots of $(\tau_{CW})^{-1}$ vs. $(1/T)$ for the ring NH resonances in d-ApTpGpCpApT in H_2O, low ionic strength, pH 7, and in 0.1 *M* NaCl-0.3 *M* phosphate-H_2O (pH 7).

At neutral pH, catalysis by 0.1 *M* phosphate (Figure 4) reflects the larger magnitude of $k_{HPO_4^{2-}}$-$[HPO_4^{2-}]$ compared to k_{OH}-$[K_W]/[H_3O^+]$, even though $k_{OH^-} \gg k_{HPO_4^{2-}}$.

The large changes in the magnitude of τ_{CW}^{-1} with temperature at low ionic strength and in the presence of phosphate are attributed to slight shifts in the ionization equilibria (eq 1 and 2) with temperature. The free energies of

$$OH^- + H_3O^+ \rightleftharpoons H_2O \quad (1)$$

$$HPO_4^{2-} + H_3O^+ \rightleftharpoons H_2PO_4^- + H_2O \quad (2)$$

these reactions predominantly account for the slopes at low ionic strength and in the presence of phosphate, respectively (Figure 4).

In the above experiments the $(AT)_{terminal}$ and $(TA)_{internal}$ residues shifted upfield as average resonances at low ionic strength, in the presence of Mg^{2+}, and of phosphate. These observations are consistent with the probability of conversion from the coil to helix, τ_{CH}^{-1}, much higher than the rate-determining transfer of the ring N proton in the coil state to water, τ_{CW}^{-1}. A knowledge of τ_{CW}^{-1} (see Figure 4) suggests that τ_{CH}^{-1} is $\gg 10^4$ at the terminal and internal base pairs.

II. Dissociation of Total Helix. The observed GC ring NH resonance originates from all species with intact GC base pairs which includes structures n_6 to n_2. (The subscript designates the number of Watson-Crick base pairs; see Table I.) Since this model assumes no chemical shift differences for the GC resonances originating from these species, no line broadening occurs for the n_6 to n_2 transition. Linewidth contributions arise from disruption of the n_2 structure. The $n_6 \rightarrow n_2$ transition, however, does determine the amount of n_2 species present in solution and hence indirectly determines the line width of the GC resonances.

It can be shown[1] that the observed line broadening of the GC resonance is equal to the equilibrium constant, $K_{6,2}$, defining the dissociation from the intact helix to the n_2 particle, times the dissociation rate of the GC pairs, $\tau_{2,0}^{-1}$. The

$$\frac{1}{T_2} - \frac{1}{T_{2H}} = K_{6,2}(\tau_{2,0})^{-1} \quad (3)$$

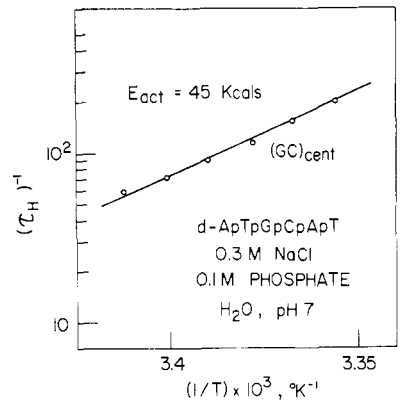

FIGURE 5: The rate of helix dissociation $(\tau_H)^{-1}$ of double-stranded d-ApTpGpCpApT in 0.3 *M* NaCl–0.1 *M* phosphate solution at pH 7 as a function of the reciprocal of the absolute temperature.

model of helix formation proposed in the literature (Applequist and Damle, 1965) involves the formation of a stable nucleus followed by a fast propagation reaction. From this model it can be easily shown (see Riesner and Romer, 1973) that the overall rate, k_d, of helix dissociation is

$$k_d = Kk_{n-1} \quad (4)$$

where K is the equilibrium constant defining the dissociation from the intact helix to the stable nucleus, which in our case is assumed to equal the n_2 structure and k_{n-1} is the dissociation rate from the nucleus to the very labile species with one base pair less. Comparison of eq 3 and 4 require that the observed line broadening of the GC ring NH resonance in d-ApTGpCpApT in the presence of phosphate is a measure of the dissociation rate of the *total* helix.

The dissociation rate constant of double helical d-ApTpGpCpApT at high salt (0.3 *M* NaCl–0.1 *M* phosphate–H_2O (pH 7)) equals 60 sec^{-1} at 20° (Figure 5). This may be compared with a dissociation rate constant of 240 sec^{-1} for the related self-complementary ribohexanucleotide duplex ApApGpCpUpU in low salt (0.05 *M* sodium cacodylate–H_2O (pH 7)) at 20° (Porschke et al., 1973). The slope in Figure 5 derived from data over a very limited temperature range yields an activation energy for the helix-coil transition of 45 kcal.

III. Nucleation and Propagation. Let us next consider the rates of formation and dissociation the central GC base pairs. The transition probability, $\tau_{2,0}^{-1}$, for disruption of the $(GC)_{central}$ base pairs in the n_2 species, i.e., the n_2 to n_0 conversion, is given by eq 3. At 20°, the excess line width of the GC resonance is 19 Hz in phosphate solution. The dissociation equilibrium constant, $K_{6,2}$, for the reaction $n_6 \rightleftarrows n_2$ is equal to the fraction of $(TA)_{int}$ base pairs in the coil form, f_c^{int}, and is equal to 0.10 at 20°. Substituting these values into eq 4 yields $\tau_{2,0}^{-1} = k_{2,0} = 6 \times 10^2$ sec^{-1} at 20°.

Knowledge of the dissociation equilibrium constant $K_{2,0} = K_{dN}$ of the GC pairs would permit the calculation of the rate constant $k_{0,2}$ for the formation of a stable nucleus, comprising the two GC pairs. As is discussed in the preceding manuscript, K_{dN} values could not be determined for these base pairs because the helix-coil exchange as monitored at the NH resonance of the $(GC)_{central}$ base pairs was found to be in intermediate exchange on a NMR time scale. We can, however, make an order of magnitude estimate of the dissociation equilibrium constant, i.e., $K_{dN} \approx 1 \times 10^{-4}$ *M*, for d-ApTpGpCpApT in H_2O, low ionic strength, pH 7. Since the dissociation rate is not very dependent on ionic strength (Porschke et al., 1973), the values of the dissociation rate and equilibrium constant can be combined to yield nucleation rate constant $k_{0,2} \approx 6 \times 10^6$ mol^{-1} sec^{-1}. This value is in agreement with what is commonly found in the literature (Riesner and Romer, 1973). The bimolecular rate of formation of the $(GC)_{central}$ base pairs, $\tau_{0,2}^{-1} = k_{0,2}[n_0]$. The single strand concentration at equilibrium, $[n_0] = f_{coil}^{cent}$ at 20° times the total strand concentration = 0.024×14 m*M* = 3.3×10^{-4} *M*. The value of $\tau_{0,2}^{-1}$ at 20° is $\approx 10^3$ sec^{-1}.

Comparison of the rates, $\tau_{0,2}^{-1}$ and $\tau_{2,0}^{-1}$, associated with the formation and dissociation of the $(GC)_{central}$ base pairs with $\tau_{2,4}^{-1}$ and $\tau_{4,6}^{-1}$, associated with formation of the internal and terminal AT base pairs (Table II) provides insight into the mechanism of helix formation.

The bimolecular nucleation reaction $\tau_{0,2}^{-1}$ ($\approx 10^3$ sec^{-1}) is slower than the unimolecular propagation reactions $\tau_{2,4}^{-1}$ and $\tau_{4,6}^{-1}$ ($\gg 10^4$ sec^{-1}). Further, the propagation rates as-

Table I: Schematic Representation of Hydrogen-Bonded Structures for the Hexanucleotide d-ApTpGpCpApT.

n_0	A		T	
	T		A	
	G		C	
	C		G	
	A		T	
	T		A	
n_1	A T		A T	
	T A		T A	
	G–C		G C	
	C G		C–G	
	A T		A T	
	T A		T A	
	a		b	
n_2	A T			
	T A			
	G–C			
	C–G			
	A T			
	T A			
n_3	A T		A T	
	T–A		T A	
	G–C		G–C	
	C–G		C–G	
	A T		A–T	
	T A		T A	
	a		b	
n_4	A–T	A T		A T
	T–A	T–A		T A
	G–C	G–C		G–C
	C–G	C–G		C–G
	A T	A–T		A–T
	T A	T A		T–A
	a	b		c
n_5	A–T		A T	
	T–A		T–A	
	G–C		G–C	
	C–G		C–G	
	A–T		A–T	
	T A		T–A	
	a		b	
n_6	A–T			
	T–A			
	G–C			
	C–G			
	A–T			
	T–A			

[1] The derivation of relationship 3 is available from the authors on request.

Table II: Summary of Rate Constants for the Nucleation and Propagation Reactions.

Nucleation	
Formation[a]	
$\tau_{0,2}^{-1} \cong 10^3$ sec^{-1}	
$k_{0,2} \cong 6 \times 10^6$ l. mol^{-1} sec^{-1}	
Dissociation[b]	
$\tau_{2,0}^{-1} = k_{2,0} \cong 6 \times 10^2$ sec^{-1}	
Propagation	
Formation[b]	
$\tau_{2,4}^{-1} = k_{2,4} \gg 10^4$ sec^{-1}	
$\tau_{4,6}^{-1} = k_{4,6} \gg 10^4$ sec^{-1}	

[a] Bimolecular. [b] Unimolecular.

sociated with formation of the AT base pairs ($\gg 10^4$ sec^{-1}) are considerably higher than the dissociation rate of the $(GC)_{central}$ base pairs, $\tau_{2,0}^{-1}$ ($= 6 \times 10^2$ sec^{-1}), a condition required to form a stable nucleus.

The formation of the double helix from separate strands of d-ApTpGpCpApT in aqueous solution can be visualized as the slow formation of a stable nucleus comprising the $(GC)_{central}$ base pairs followed by rapid propagation involving the $(TA)_{internal}$ and $(AT)_{terminal}$ base pairs.

Acknowledgment

The expert technical assistance of Miss L. L. Canuel is acknowledged.

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Identification of a Unique Ethidium Bromide Binding Site on Yeast $tRNA^{Phe}$ by High Resolution (300 MHz) Nuclear Magnetic Resonance†

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ABSTRACT: The binding of ethidium bromide to yeast $tRNA^{Phe}$ has been studied by high resolution (300 MHz) proton nuclear magnetic resonance. Under appropriate experimental conditions one ethidium bromide is bound to each tRNA and two resonances from ring NH protons are shifted upfield. These observations taken in conjunction with the assignments of the low-field spectrum of yeast $tRNA^{Phe}$ show that the unique ethidium bromide binding site is located between base pairs AU_6 and AU_7 of the amino acid acceptor stem. This information should be of value in understanding the way in which ethidium bromide binding alters the biochemical properties of the tRNA molecules.

Ethidium bromide is one of a number of dyes and drugs known to strongly interact with polynucleotides (Waring, 1965; Bauer and Vinograd, 1968) and the physical (Hudson et al., 1969; Bittman, 1969), chemical (Harbers et al., 1972), and biological (Perlman and Mahler, 1971; Lurquin and Buchet-Mahieu, 1971) consequences of this interaction have been the subject of numerous investigations. Antiviral and antibacterial properties have been observed for ethidium bromide (Dickinson et al., 1953) and it is known that ethidium bromide inhibits nucleic acid synthesis (Tomchick and Mandel, 1964), as well as DNA and RNA polymerase activity (Elliott, 1963; Waring, 1964). The observations of in vivo biological activity have raised interesting questions about the way in which ethidium bromide affects the properties of DNA and RNA. It is already known that ethidium bromide binds strongly to double-stranded DNA and RNA (Le Pecq and Paoletti, 1967; Aktipis and Martz, 1974), by intercalating between adjacent base pairs in the double helix (Waring, 1964; Kreishman et al., 1971), but more information about the mechanism of binding and the local site requirements is needed to understand how ethidium

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