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A Comparative Study of Ethidium Bromide Complexes with Dinucleotides and DNA: Direct Evidence for Intercalation and Nucleic Acid Sequence Preferences†

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ABSTRACT: The relative fluorescence quantum yields of ethidium bromide solutions with deoxyribodinucleoside monophosphates have been measured and found to correlate with similar data for the binding of ethidium to DNA. The fluorescence lifetime of ethidium has been measured both in solution and in *polycrystalline 2:2 ethidium complexes* with the ribodinucleoside monophosphate C-G. In aqueous solutions containing an excess of nucleotide, the experimental decay curve could be accurately fit using a single exponential decay with a lifetime of 23 ns, whereas the cocrystalline complexes exhibited two exponential decays with lifetimes of 4 and 10 ns. Detailed ^{1}H nuclear magnetic resonance experiments have shown that in the presence of excess nucleotide the solution complex is one in which ethidium is intercalated between two Watson-Crick G·C base pairs. The observation of two spectroscopically distinct ethidiums in the 2:2 cocrystalline complexes is consistent with the structure of the 2:2 5-iodoC-G complexes determined by X-ray cocrystallographic techniques [Tsai, C. C., Jain, S. C., and Sobell, H. M. (1977), *J. Mol. Biol. 114,* 301–315] in which one ethidium was intercalated between two G·C base pairs while the second ethidium was stacked on the end of the miniature double-helical complex. We have also observed that ethidium will stack on the end of the miniature double-helical complexes at both the dinucleotide (this manuscript) and the deoxytetranucleotide levels [Kastrup, R. V., Young, M. A., and Krugh, T. R. (1978), *Biochemistry 17,* following paper in this issue] when there is an excess of ethidium compared to the number of favorable intercalation sites. Thus, the combined use of optical and nuclear magnetic resonance spectroscopic studies has provided a link between the solution studies and the solid-state studies. Ethidium exhibits a clear preferential binding to pyrimidine–purine deoxydinucleotides as compared to their purine–pyrimidine sequence isomers. ^{1}H nuclear magnetic resonance experiments show that ethidium forms a miniature intercalated complex with either the self-complementary deoxydinucleotides pdC-dG, dC-dG, and pdT-dA or with a mixture of the complementary dinucleotides dT-dG plus dC-dA. The self-aggregation of ethidium was studied by monitoring the concentration dependence of the phenanthridinium protons. Ethidium aggregation can be adequately represented by dimer formation with a dimerization constant $K_d \simeq 95$ M^{-1} at 3 °C in 0.1 M NaCl. The dimerization constant was also measured at 25 °C ($K_d \simeq 70$ M^{-1}) and at both 3 and 25 °C in the absence of salt ($K_d \simeq 64$ and 48 M^{-1}, respectively). A photon-counting spectrofluorimeter was used to measure the binding of ethidium bromide to calf thymus DNA by itself and in the presence of actinomycin D and actinomine. An analysis of the binding data indicates that ethidium bromide and actinomycin D do not compete for the same binding sites (at low r values), which suggests that these drugs preferentially bind to different sequences of DNA, as suggested by the model studies with the oligonucleotides. On the other hand, ethidium bromide does compete with actinomine, which is again consistent with the visible spectral titrations of the model compounds since actinomine appears to bind equally well to both pdG-dC and pdC-dG.

The nature of the interaction of ethidium bromide (Figure 1), a trypanocidal drug, with nucleic acids has been examined by a variety of techniques over the past 15 years (Le Pecq et al., 1964; Waring, 1965; Le Pecq, 1971; Krugh et al., 1975; Krugh and Reinhardt, 1975; and references therein). It is generally recognized that the strong mode of binding of ethidium bromide to double-stranded nucleic acids results in the intercalation of the planar phenanthridinium ring between adjacent base pairs on the double helix. Evidence obtained from the various physicochemical studies of ethidium–DNA and ethidium–RNA complexes supports the intercalation model for ethidium bromide as proposed by Fuller and Waring (1964). Direct evidence for the intercalation of ethidium into nucleic acids has been obtained from studies of the solution complexes of ethidium with the deoxydinucleotide and ribo-

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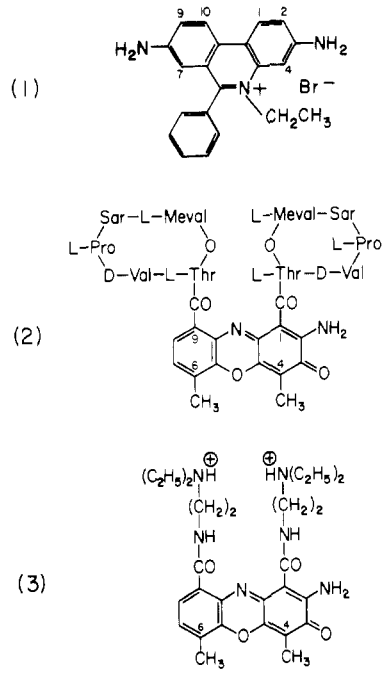

FIGURE 1: Structural formulas of (1) ethidium bromide, (2) actinomycin D, and (3) actinomine.

dinucleoside monophosphates (Krugh et al., 1975; and Krugh and Reinhardt, 1975), as well as subsequent X-ray crystallographic studies of ethidium complexes with 5-iodoU-A and 5-iodoC-G in the solid state (Tsai et al., 1975, 1977; Jain et al., 1977), and more recent solution studies of ethidium binding to deoxytetranucleotides (Patel and Canuel, 1976; Kastrup et al., 1978).

Analysis of the binding of ethidium to DNA and RNA provides only indirect evidence concerning the question of whether ethidium binds to the various sequences available as intercalation sites on the nucleic acid with the same or with significantly different binding constants. The preferential binding of ethidium is an important area which will be addressed in this and the following paper in this issue. The results of our solution studies with the deoxydinucleotides are presented in this paper, along with competitive binding studies of ethidium bromide, actinomycin D, and actinomine to polydeoxynucleotides. These data support our previous observation that ethidium bromide binds more strongly to pyr(3′-5′)pur[1] sequences than to pur(3′-5′)pyr sequences. We propose that ethidium has a range of binding constants for the ten unique intercalation sites on both the ribo and deoxyribo double-stranded nucleic acids.

Experimental Section

Ethidium bromide was purchased from Sigma Chemical Co. Actinomycin D and actinomine were kindly supplied by Dr. S. K. Sengupta of the Sidney Farber Cancer Center, Boston, Mass. Drug concentrations were determined spectrophotometrically using the following extinction coefficients: ethidium bromide, ϵ_{480} 5860 (K. R. Lee and T. R. Krugh, unpublished data; see also Wakelin and Waring, 1974; Bresloff and Crothers, 1975); actinomycin D, ϵ_{425} 23 500 (Hyman and Davidson, 1971); actinomine, ϵ_{445} 22 500 (Blau and Bittman, 1975). The ethidium bromide was dissolved in aqueous solution and lyophilized to remove trace ethanol impurities. Actinomine was used without further purification. The actinomycin D was purified by elution with ethyl acetate on an aluminum oxide column (Woelm, neutral activity IV, 10% H_2O) packed in benzene. The actinomycin D fractions were collected, and the ethyl acetate was removed by evaporation. This procedure accomplished the removal of 90–95% of the fluorescence which was initially present in the actinomycin D sample. The question of actinomycin D fluorescence has been recently studied by Chinsky and Turpin (1977). Calf thymus DNA (type I) and the ribodinucleoside monophosphate C-G were purchased from Sigma Chemical Co. The deoxydinucleotides and deoxydinucleoside monophosphates were purchased from either Collaborative Research, Inc., or from P-L Biochemicals. Dinucleotides were treated with Chelex-100 (Bio-Rad), filtered through an 8-μm Millipore filter, and lyophilized. Unless otherwise noted, all of the dinucleotide solutions were dissolved in a 5 mM phosphate buffer in either H_2O solution (pH 7.0) or D_2O solution (pH meter reading 7.0, pD 7.4). The DNA was purified following the procedure of Müller and Crothers (1975), and all DNA solutions were prepared in 50 mM Tris-HCl[1] (pH 7.5), 0.2 M NaCl. The concentrations of the DNA solutions are stated in terms of the concentration of nucleotide phosphorus, using an extinction coefficient of ϵ_{260} 6600. The concentrations of the dinucleotides, the visible absorption titrations, the nuclear magnetic resonance experiments, and the static fluorescence experiments were measured or performed in a manner described previously (Krugh and Reinhardt, 1975).

The competitive-binding fluorescence titrations were performed on an SLM scanning photon-counting spectrofluorimeter. Excitation was at 546 nm, and emission was monitored at 590 nm. Titrations were performed in a 1-cm fluorescence cuvette with a Teflon stopper. Two-milliliter samples of DNA stock solution were used; the concentration of DNA was kept constant during the titration by simultaneously adding, along with the ethidium aliquots, equal aliquots of a DNA solution at twice the stock concentration. All samples were thoroughly mixed by inversion and were allowed to equilibrate for 5 min at 21 °C.

Fluorescence Lifetime Measurements. Ethidium bromide and C-G were cocrystallized from approximately equimolar mixtures of the drug and dinucleotide in 5 mM D_2O–phosphate buffer (pD 7.4). Two sets of cocrystalline precipitates were prepared independently: crystalline sample I precipitated immediately from a solution mixture which contained ~7 mM C-G and ~7.5 mM ethidium bromide at room temperature, and crystalline sample II precipitated gradually from a 1:1 (~3.7 mM) solution mixture which was stored at ~2 °C for 12 h. The ethidium bromide–C-G crystals appeared as a dark reddish-orange precipitate. After drawing off the supernatant, small quantities of the cocrystalline precipitates (~7 μL) were washed thoroughly to remove the residual mother liquor and then dissolved in 0.1 N HCl for analysis of the C-G [ϵ_{276} (0.1 N HCl) 1.98×10^4] and ethidium bromide [ϵ_{276} (0.1 N HCl) 2.9×10^4; ϵ_{480} (pH 7) 5860] concentrations by UV-visible spectrophotometry. In both crystals I and II, the C-G/ethidium bromide ratio was 1.0:1.0 (or 2:2) even though the C-G/ethidium ratio of the mother liquors was quite different.

Samples for fluorescence lifetime measurements were prepared as described previously (Langlois et al., 1975; Reinhardt, 1977). The fluorescence lifetimes were measured with a single photon-counting instrument built in the laboratory of Dr. Bruce Love. This equipment is similar to that previously described (Yguerabide, 1972). A free-running air flash lamp operating at a rate of ~25 000 Hz was used to excite the samples. A Corning S-7-60 filter was used in the excitation

[1] Abbreviations used: pyr, pyrimidine; pur, purine; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

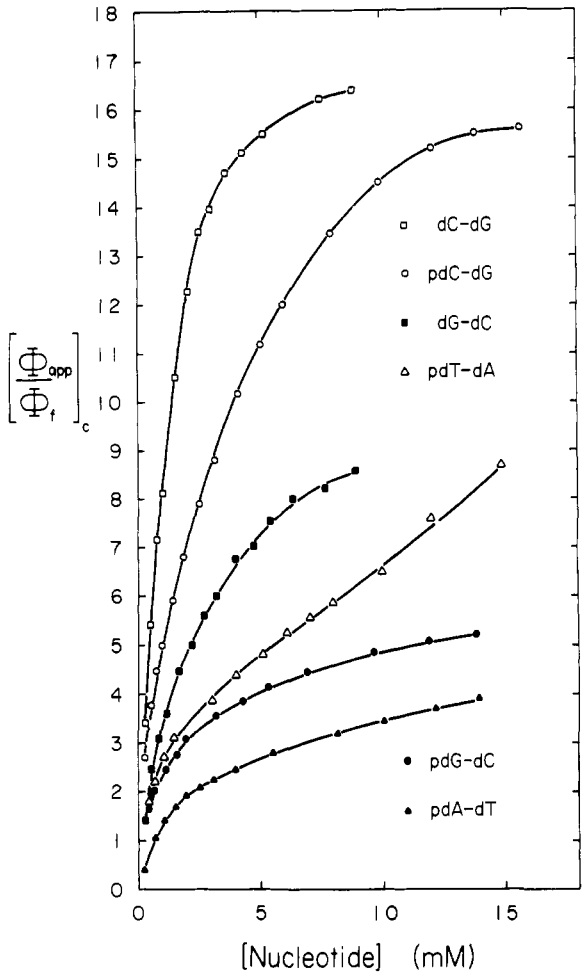

FIGURE 2: Fluorescence enhancement of ethidium bromide as a function of increasing self-complementary deoxydinucleotide concentrations (at 0 °C). The ethidium concentration at the start of the titrations was 2.7 × 10^{-4} M. Excitation wavelength was 546 nm and the emission was monitored at 590 nm.

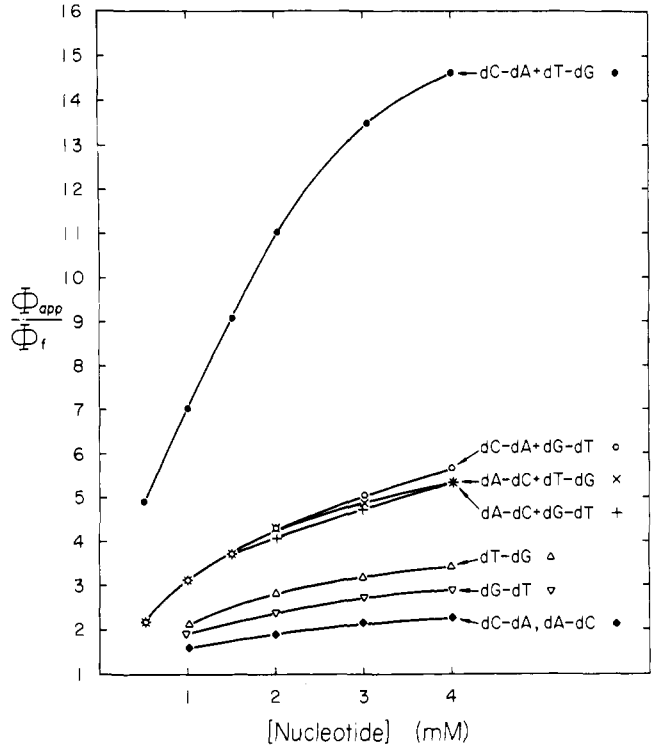

FIGURE 4: Fluorescence titrations of ethidium bromide with binary mixtures of several non-self-complementary deoxydinucleotides (at 2 °C). The fluorescence enhancement of ethidium is plotted as a function of increasing concentrations of the various dinucleotides. The nucleotide concentration is given in terms of the individual concentrations of each dinucleotide in the solution. The concentration of ethidium bromide at the start of each titration was 2.6 × 10^{-4} M. Excitation wavelength was at 546 nm, and emission was monitored at 590 nm.

beam and a Corning 3-69 filter was placed before the entrance to the emission slits. Multiple photon counts were minimized by "energy windowing" (Isenberg, 1975). The experimental decay data were deconvoluted from the lamp pulse by computer analysis to give single or double exponential fits using the method of moments (Yguerabide, 1972).

Results

Fluorescence and Visible Titration

The fluorescence of ethidium bromide is significantly increased upon binding to double-stranded nucleic acids (LePecq, 1971). The relative fluorescence quantum yield ratios, which are corrected for free ethidium, $(\phi_{app}/\phi_f)_c$, for complexes of ethidium bromide with the self-complementary deoxydinucleotides are plotted as a function of increasing dinucleotide concentration in Figure 2. A comparison of the shape of the fluorescence titration curves of sequence isomer pairs in Figure 2 shows a clear preference for ethidium to bind more strongly to the pyrimidine–purine sequence deoxydinucleotides when compared to the corresponding purine–pyrimidine sequence isomers. The fluorescence enhancement of ethidium bound to dC-dG and pdC-dG in solution at high deoxydinucleotide concentrations (Figure 2) is approaching the fluorescence quantum yield ratio corresponding to an ethidium–DNA complex for which $(\phi_{app}/\phi_f)_c = 21$ (LePecq and Paoletti, 1967) and is nearly identical to the fluorescence increase for ethidium intercalated into poly(dG-dC) for which $(\phi_{app}/\phi_f)_c = 15$ (Pohl et al., 1972).

The change in absorbance of ethidium bromide at 546 nm was also monitored at each point in the fluorescence titrations of ethidium bromide with the self-complementary deoxydinucleotides. These data were qualitatively similar to the previously reported spectral titrations of ethidium with the self-complementary ribodinucleoside monophosphates (Krugh and Reinhardt, 1975). A comparison of the shapes of the deoxydinucleotide binding curves for identical sequence isomer pairs such as dC-dG vs. dG-dC, pdC-dG vs. pdG-dC, and dT-dA vs. dA-dT indicates a preferential binding of ethidium bromide to the deoxydinucleotides which have a pyr(3′–5′)pur sequence when compared to their corresponding pur(3′–5′)pyr sequence isomers. The titration curves for the pyrimidine–purine sequence deoxydinucleotides also have a definite sigmoidal shape (which is more pronounced for the dC-dG and pdC-dG titration curves) at nucleotide concentrations below 0.25 mM, which is indicative of cooperative binding.

The changes in the absorption spectra at 546 nm and the changes in the relative fluorescence quantum yield ratios, ϕ_{app}/ϕ_f, of ethidium bromide for mixtures of ethidium with the non-self-complementary deoxydinucleotides are presented in Figures 3 (in the supplementary material) and 4, respectively. The absorption and the fluorescence results parallel the circular dichroism results presented previously (Krugh et al., 1975). Among the binary mixtures studied, only the solution complex of ethidium with the complementary pyrimidine (3′–5′)purine deoxydinucleotide mixture, dC-dA + dT-dG, exhibits the visible absorption, induced circular dichroism, and enhanced fluorescence characteristics observed for ethidium

TABLE I: Wavelength of Maximum Extinction Coefficient in the Visible Absorption Spectra of Ethidium Bromide–Deoxydinucleotide Solutions.[a]

deoxydinucleotide	λ_{max} (nm)
dC-dG	520
dG-dC	509
pdC-dG	521
pdG-dC	502
pdT-dA	511
pdA-dT	487
dC-dA	486
dA-dC	485
dG-dT	485
dT-dG	488
dC-dA + dT-dG	518
dA-dC + dG-dT	499
dC-dA + dG-dT	499
dA-dC + dT-dG	498
calf thymus DNA	520[b]

[a] Measured from the absorption spectrum of the respective ethidium bromide–dinucleotide solutions; 5 mM phosphate buffer. pH = 7.0, T = 0–2 °C, ethidium bromide concentration was 2.6×10^{-4} M. See Reinhardt (1977) for additional details. [b] Waring, 1965; Le Pecq and Paoletti, 1967.

bromide complexes with double-stranded nucleic acids. Ethidium binds to all of the deoxydinucleotide sequences and their binary mixtures, as evidenced by the changes observed in the absorbance and fluorescence of ethidium in these solutions. However, the complex of ethidium which is formed in the dC-dA plus dT-dG solution appears to be unique, in that the spectral properties of ethidium in this solution most closely parallel those properties which are associated with an intercalated ethidium bromide–nucleic acid complex. Thus, these data illustrate the role of complementarity and the binding preference of ethidium for pyrimidine–purine sequence dinucleotides.

The wavelengths of the maximum visible absorbance, λ_{max}, of several different ethidium bromide–deoxydinucleotide solutions are presented in Table I. The data in Table I provide a clear illustration that the self-complementary dinucleotides with a pyrimidine–purine sequence exhibit a much larger bathochromic shift in the visible spectra of these solutions as compared to the spectra of the corresponding ethidium solutions with the pur(3′–5′)pyr sequence. The data in Table I for the mixtures of complementary nucleotides show that only the dC-dA plus dT-dG solution with ethidium bromide exhibits a λ_{max} indicative of the intercalated complex under the present experimental conditions.

Fluorescence Lifetime Measurements. The increase in the fluorescence intensity of ethidium bromide when it intercalates into double-helical nucleic acids is accompanied by a significant increase in the fluorescence lifetime, τ. For example, the fluorescence lifetime of ethidium bromide increases from 1.8 ns for free ethidium to approximately 23 or 19 ns for complexes of ethidium bromide with double-stranded DNA or RNA, respectively (Burns, 1969, 1971). It is generally accepted that these lifetimes correspond to the intercalated ethidium bromide. Olmstead and Kearns (1977) have recently proposed a mechanism for the enhancement of ethidium fluorescence upon intercalation, which involves a reduction in the rate of excited-state proton transfer from the ethidium amino groups to solvent molecules. At low nucleotide to drug ratios and low ionic strengths, a shorter fluorescence component is also observed which has been attributed to the secondary electrostatic binding of ethidium to the outside of the nucleic acid helix (Olmstead and Kearns, 1977; Tao et al., 1970). We might therefore expect that an analysis of the fluorescence lifetimes of the ethidium–dinucleotide complexes may provide a sensitive probe to the geometry of these complexes. Previous X-ray crystallographic studies of the solid-state complexes of ethidium with 5-iodoC-G (and 5-iodoU-A) showed that ethidium was both intercalated and stacked over the miniature double-helical complex in these 2:2 5-iodoC-G (or 5-iodoU-A)/ethidium bromide cocrystals (Tsai et al., 1975, 1977; Jain et al., 1977). We have measured the fluorescence lifetimes of ethidium bromide in aqueous solutions with C-G as well as the fluorescence decay properties of the 2:2 cocrystalline C-G–ethidium bromide complex. We would expect the ethidium molecules in the solid-state 2:2 complex to give rise to two distinct fluorescence lifetimes corresponding to the two different environments of ethidium (intercalated plus stacked), unless there is efficient energy transfer among the ethidiums in the crystal.

Static fluorescence measurements were made on the microcrystalline ethidium bromide–C-G samples prior to the fluorescence lifetime measurements in order to establish that the observed fluorescence emission was in fact due to the crystals and not due to the residual mother liquor in which they were suspended. An 86-fold increase was observed for the fluorescence of the capillary containing the packed microcrystals as compared to an identical volume of the corresponding supernatant (Reinhardt, 1977). We therefore conclude that the emission from the capillary containing the crystals originates from the ethidium bromide–C-G cocrystalline complexes. The fluorescence decay parameters analyzed from the experimental single-photon counting data for the ethidium bromide–C-G cocrystalline complex and an ethidium–C-G solution are presented in Table II. The fluorescence decay data of the crystals was best fitted to a *double*-exponential decay expression with two distinct fluorescence lifetimes; the shorter lifetime was equal to 4.5 ns (α_1 = 0.55–0.58) and the longer lifetime was equal to 10–12 ns (α_2 = 0.42–0.45). The preexponential factors, α, give an approximation of the fractional populations of each fluorescent component. The fluorescence decay of an aqueous solution of ethidium bromide with C-G at high nucleotide to drug ratios ([C-G] = 6.9×10^{-3} M; [EthBr] = 1.3×10^{-4} M, Table II) could be adequately represented by a *single*-exponential decay with a fluorescence lifetime of approximately 23 ns. Attempts at double-exponential fits to this data yielded a zero coefficient for the short-lived component. Furthermore, it is expected that the presence of a second lifetime component (in addition to the 23-ns component) could be detected by using different decay analysis times which should result in different apparent lifetimes (e.g., see Olmstead, and Kearns, 1977). Truncation of the fluorescence lifetime analysis at 82 ns gave identical lifetimes (within an estimated experimental error of ±0.3 ns) as did extending the decay analysis out to 124 ns. For aqueous solutions of ethidium bromide with C-G at lower nucleotide to drug ratios (supernatant I, Table II), we observed two fluorescence lifetimes, a major component with a lifetime of 5.3 ns and a minor component with a lifetime of 17.3 ns.

Proton Magnetic Resonance

In view of the recent interest in the self-association of intercalating dyes (Kreishman et al., 1971; Subramanian et al., 1971; Turner et al., 1974) and, in particular, its effect on the induced shifts of proton resonances observed in NMR experiments of drug–nucleotide complexes (for example, see Krugh and Chen, 1975), we have reexamined the concentration dependence of the ethidium proton chemical shifts using pulsed

TABLE II: Fluorescence Lifetimes of Ethidium Bromide: C-G Complexes in Crystals and in Solution (T = 23 °C).

sample	$S(t)$ parameters[a]				[C-G] (M)	[EthBr] (M)
	α_1	τ_1 (ns)	α_1	τ_2 (ns)		
crystals I	0.58	5.1	0.42	11.6	*b*	*b*
crystals II	0.55	4.0	0.45	10.2	*b*	*b*
supernatant I	0.87	5.3	0.13	17.3	6.8×10^{-4}	1.1×10^{-3}
aqueous solution	1.0	22.8	*c*	*c*	6.9×10^{-3}	1.3×10^{-4}

[a] Decay curves were fitted by the double-exponential function: $S(t) = \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2}$; the fluorescence lifetimes (τ_1, τ_2) and amplitudes (α_1, α_2) are the best computer-calculated decay parameters obtained from deconvolution of the experimental decay with the lamp pulse by the method of moments. [b] Estimated to be $\sim 12 \times 10^3$ M. [c] The experimental decay data could be accurately fit to a single-exponential decay function: $S(t) = \alpha_1 e^{-t/\tau_1}$.

TABLE III: Ethidium Bromide Dimerization Parameters.

temp (°C)	no salt[a]				0.1 M NaCl[a]			
		δA (ppm)	δA_2 (ppm)	K_d (M^{-1})		δA (ppm)	δA_2 (ppm)	K_d (M^{-1})
3	H(1), H(10)	8.64 ± 0.02	7.29 ± 0.04	67 ± 9	H(1), H(10)	8.63 ± 0.03	7.17 ± 0.03	101 ± 12
	H(7)	6.66 ± 0.01	5.95 ± 0.03	60 ± 8	H(7)	6.65 ± 0.01	5.87 ± 0.02	85 ± 11
25	H(1), H(10)	8.68 ± 0.02	7.34 ± 0.04	48 ± 6	H(1), H(10)	8.69 ± 0.02	7.25 ± 0.03	75 ± 7
	H(7)	6.66 ± 0.01	5.98 ± 0.02	48 ± 5	H(7)	6.66 ± 0.01	5.90 ± 0.02	67 ± 6

[a] All samples were dissolved in 5 mM phosphate buffer in D_2O; pH meter = 7.0.

Fourier transform NMR techniques (Figure 5, in supplementary material). Dimerization constants for the self-association via vertical stacking of the phenanthridinium rings of ethidium bromide are listed in Table III. A discussion of the results is included in the caption for Figure 5 (in the supplementary material).

The proton magnetic resonance spectra of ethidium bromide, the deoxydinucleoside monophosphate dC-dG, and a 2:1 dC-dG-ethidium bromide solution are shown in Figure 6 (in supplementary material). In Figure 7, the induced chemical shifts of ethidium protons that result from complex formation with dC-dG are plotted as a function of the dC-dG/ethidium bromide ratio. Note that the H(1), H(10) proton resonances are shifted upfield ~0.5 ppm, while the H(7) resonance moves upfield by at least 0.9 ppm as a result of the formation of a dC-dG-ethidium bromide complex. The phenyl protons do not appear to be sensitive to complex formation, as indicated by the relatively small shifts of these resonances during the titration.

The direction and magnitude of the induced chemical shifts in Figure 7 for ethidium protons on opposite sides of the phenanthridinium ring are only consistent with the vertical stacking of the nucleotide bases with the phenanthridinium ring. From the shape of the CH_3, H(2), H(4), H(9), and extrapolated H(7) proton curves in Figure 7, it is apparent that the chemical shifts of the ethidium protons begin to level off at a nucleotide to drug ratio of ~2:1, thus suggesting a stoichiometry of *two* dC-dG molecules for each ethidium bromide molecule in the complex. It is important to recall that in the millimolar concentration range of the present experiments the deoxydinucleotides exist predominantly as monomers in the absence of ethidium bromide (e.g., see Young and Krugh, 1975). Any hydrogen bonding of the self-complementary dinucleotides must therefore result from the formation of a dC-dG-ethidium bromide complex.

Further evidence for a dC-dG-ethidium bromide complex involving two dC-dG molecules has been obtained from the proton Fourier transform spectrum of a solution containing 0.4 M pdC-dG and 0.2 M ethidium bromide in H_2O (Figure 8). The experimental techniques used in this laboratory for recording NMR spectra in H_2O have been previously described (Mooberry and Krugh, 1975; Krugh and Schaefer 1975) and provide the opportunity of observing the hydrogen-bonded NH protons of the dinucleotides. The broad low-field resonance at 11.95 ppm in Figure 8 is assigned to the guanine NH proton. In the absence of ethidium bromide this resonance cannot be observed due to the rapid exchange of the NH proton with the solvent water protons. Hydrogen-bond formation results in a slower exchange rate for this proton, so that the observation of the NH resonance in the pdC-dG plus ethidium bromide solution clearly indicates the formation of hydrogen-bonded base pairs between the complementary guanine and cytosine bases on opposite nucleotides. Both the chemical shift and the relatively small intensity of this resonance [which results from transfer of saturation effects (e.g., see Krugh and Young, 1975)] make us confident that this is the NH resonance from a G-C base pair as opposed to a NH_2 resonance.

The proton magnetic resonance spectra of solutions of ethidium bromide with dG-dC have also been examined. A 3:1 dG-dC/ethidium bromide solution at 25 °C (data not included; see Reinhardt, 1977) shows upfield shifts of all of the phenanthridinium ring protons when compared to the spectrum of free ethidium bromide, but the magnitudes of these shifts are significantly less than the induced shifts observed in the 2:1 dC-dG/ethidium bromide spectrum (Figure 7).

A comparison of the proton magnetic resonance spectra of ethidium complexes with dT-dA and dA-dT is shown in Figure 9 (in supplementary material) and the proton magnetic resonance spectra of ethidium solutions with dC-dA, dT-dG, and a mixture of dC-dA plus dT-dG are presented in Figure 10 (in supplementary material). Only mixtures of ethidium bromide with complementary pyr(3′-5′)pur sequence dinucleotides showed upfield shifts of the ethidium resonances of magnitude similar to that observed for the dC-dG/ethidium bromide system. The other solutions which contained mixtures of complementary and noncomplementary dinucleotides with ethidium bromide showed only small chemical-shift changes of the ethidium resonances in their respective proton magnetic resonance spectra (Reinhardt, 1977). A discussion of these results is included in the captions to Figures 9 and 10.

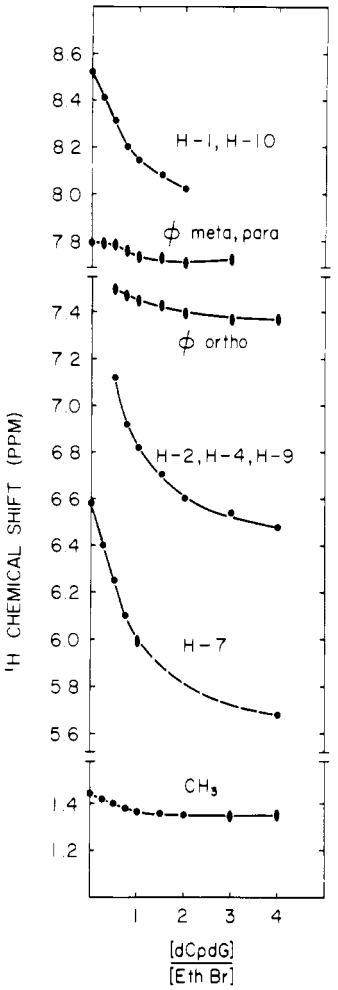

FIGURE 7: Chemical shifts of selected ethidium bromide groups plotted as a function of the nucleotide to drug ratio for dC-dG at 25 °C. Chemical shifts were measured relative to TSP from the spectra of solutions containing 1.6 mM ethidium bromide titrated with dC-dG in 5 mM D_2O–phosphate buffer (pD 7.4). The data points correspond to the peak positions as monitored at the center of the respective proton clusters or multiplets, with the exception of the H(2), H(4), and H(9) group assignment which was monitored at the furthermost downfield peak within this cluster of resonances. The individual resonances for the H(2), H(4), and H(9) protons were not resolvable at 100 MHz.

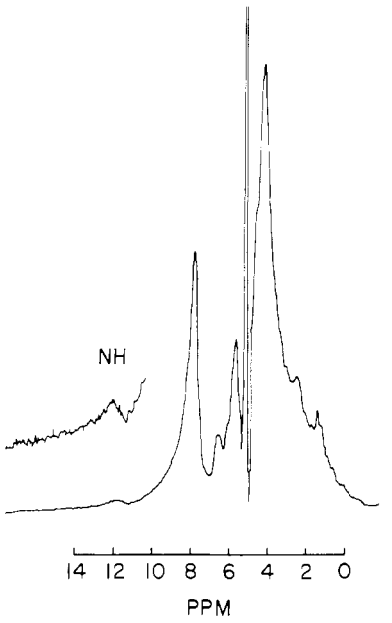

FIGURE 8: 100-MHz proton FT NMR spectrum of the pdC-dG–ethidium bromide complex in aqueous solution. The ethidium bromide concentration was 0.02 M with 0.04 M pdC-dG in an H_2O solvent (pH 7.0), 3 °C. The 180°-τ-90° pulse sequence was used to minimize the solvent resonance. The free-induction decay was accumulated 1500 times before Fourier transformation: τ = 0.48 s; pulse repetition time = 1.5 s; and a homogeneity spoiling pulse was applied for 0.3 s after the 180° pulse. The inset shows the low-field region with a sixfold gain increase. The NH resonance is 11.95 ppm from sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$.

Competitive Binding

Although ethidium bromide does not have a requirement for any particular *base* in binding to DNA (Waring, 1965; LePecq and Paoletti, 1967), it does show a definite binding preference in forming complexes with C-G, dC-dG, and other pyrimidine–purine sequence dinucleotides, as indicated by the visible absorbance, fluorescence, and proton magnetic resonance data presented in this paper (see also Krugh et al., 1975; Krugh and Reinhardt, 1975). Previous studies on the solution complexes of actinomycin D (Figure 1) with the deoxydinucleotides (Krugh, 1972; Schara and Müller, 1972; Patel, 1974; Krugh and Chen, 1975; Davanloo and Crothers, 1976; Krugh et al., 1977; Reinhardt and Krugh, 1977) have shown that actinomycin D will form a miniature intercalated complex with pdG-dC and with a mixture of pdG-dT plus pdA-dC, which are purine–pyrimidine sequence dinucleotides. Actinomycin D forms a stacked complex with the pyrimidine–purine sequence dinucleotides pdC–dG. We have therefore examined the binding interactions of ethidium bromide with DNA in the presence of actinomycin D, in the hope of providing information about the similarities or differences in the nature of the binding sites of these drugs to DNA.

Fluorescence Scatchard plots for the binding of ethidium bromide to calf thymus DNA in the absence and presence of actinomycin D are shown in Figure 11. Spectrofluorimetric analysis of the binding of ethidium bromide to DNA by the use of Scatchard plots has been previously described (LePecq and Paoletti, 1967). The Scatchard plot for the ethidium bromide–DNA interaction in the absence of actinomycin D is linear in the range of r values from 0.04 to 0.18 with an apparent binding constant of 3.1×10^5 M^{-1} in 0.2 M NaCl at 21 °C (Figure 11). There is approximately one binding site per five nucleotides, i.e., approximately one intercalation site every other base pair as determined from the extrapolated value of r at saturation (r = 0.2). An extrapolated literature value for the binding of ethidium bromide to calf thymus DNA in 0.2 M NaCl is 3.4×10^5 M^{-1} (23 °C) (LePecq and Paoletti, 1967). However, see below for an alternative analysis of the binding data. Figure 11 clearly shows that the binding parameters of the ethidium bromide–DNA interaction are modified in the presence of actinomycin D, as was first observed by LePecq and Paoletti (1967). Control experiments have indicated that the fluorescence characteristics of both free and bound ethidium bromide are unchanged in the presence of purified actinomycin D at the concentrations in which the present competitive binding experiments were performed (Reinhardt, 1977). The ethidium bromide binding at low r values ($r < 0.12$) in the presence of actinomycin D (DNA/actinomycin D = 5, Figure 11) is characterized by a Scatchard plot with an apparent binding constant of 2.9×10^5 M^{-1} and an extrapolated value of 0.15 for the number of binding sites per nucleotide phosphate. These data fit the model for *noncompetitive* binding, in which the association constant of ethidium bromide is not affected, but the number of binding sites is reduced. This same phenomenon has been observed for the simultaneous binding of actinomycin D to DNA in the presence of netropsin (Wartell et al., 1975) and polylysine

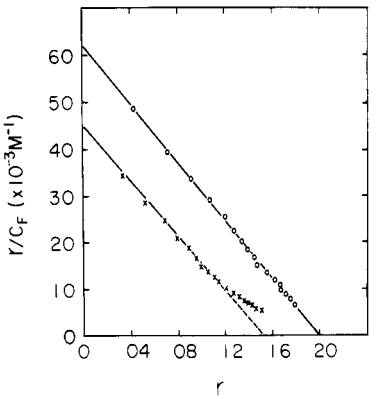

FIGURE 11: Competition of actinomycin D with ethidium bromide for binding sites on DNA. Fluorescence Scatchard plots of EthBr (concentration 1.0-29.1 μM) in 50 mM Tris-HCl (pH 7.5), 0.2 M NaCl buffer (O) and in the presence of Act D, [DNA·P]/[Act D] = 5 (x).

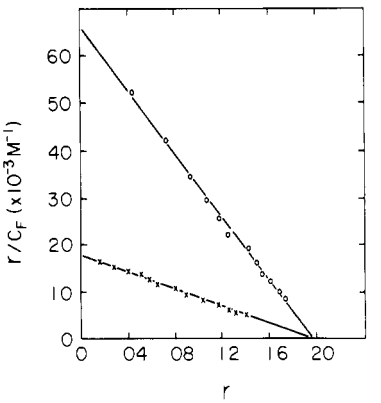

FIGURE 12: Competition of actinomine with ethidium bromide for binding sites on DNA. Fluorescence Scatchard plots of ethidium bromide (concentration 1.0-28.3 μM) bound to calf thymus DNA ([DNA·P] = 3.5 μM) in 50 mM Tris-HCl (pH 7.5), 0.2 M NaCl buffer (O) and in the presence of actinomine, ([DNA·P]/[actinomine]) = 0.5.

(Carroll and Botchan, 1972). In the latter case, the apparent noncompetitive behavior was attributed to an irreversibly bound competitor, but this possibility can be excluded from consideration in our present experiments, since actinomycin D binds to calf thymus DNA in a reversible fashion with an apparent binding constant ($K_{app} = 3 \times 10^6$ M^{-1}) (Müller and Crothers, 1968) which is only an order of magnitude greater than that of ethidium bromide ($K_{app} = 3 \times 10^5$ M^{-1}) under conditions of comparable ionic strengths. The length of the DNA helix which is covered by an intercalated actinomycin D molecule appears to be four to six base pairs (Krugh et al., unpublished data; Müller and Crothers, 1968; Wells and Larson, 1970; Wartell et al., 1975). Therefore, it is conceivable that the number of potential sites for the binding of ethidium bromide molecules to DNA could be reduced by steric interference from bound actinomycin D even under conditions of noncompetitive binding, since ethidium is capable of binding at an intercalation site approximately every other base pair. At the higher r values ($r > 0.12$), the nonlinearity in the Scatchard plot (Figure 11) indicates that the apparent binding constant of ethidium is being reduced in the presence of actinomycin D and that the extrapolated number of binding sites at saturation is increasing.

Actinomine (Figure 1), an analogue of actinomycin D which contains the phenoxazine moiety but has *N,N*-diethylenediamine side chains replacing the cyclic pentapeptide rings, binds very strongly to DNA in solutions of low ionic strength (Müller and Crothers, 1968). Intercalation has also been proposed as the mechanism for this interaction (Mt iüller and Crothers, 1968, Wawra et al., 1970; Seela, 1971; Zipper and Bünemann, 1975). The Scatchard plot for the binding of ethidium bromide to calf thymus DNA in the absence and presence of actinomine (DNA/actinomine = 0.5) is shown in Figure 12. The apparent binding constant of ethidium bromide is decreased in the presence of actinomine, but the number of binding sites at saturation is unaffected. These binding characteristics are consistent with the model for competitive inhibition. Analysis of the data in Figure 12, assuming competitive inhibition (Tanford, 1961), gives a binding constant of 3.5×10^5 M^{-1} for actinomine in 0.2 M NaCl, which is in reasonable agreement with the value ($\sim 10^6$ M^{-1}) determined from the absorption binding isotherms for the actinomine-DNA interaction at comparable salt concentrations (Müller and Crothers, 1968). The competitive binding of ethidium bromide and actinomine with DNA as observed from the data in Figure 12 is not surprising in view of our preliminary studies on actinomine-deoxydinucleotide model complexes. The shapes of the visible absorbance titrations of actinomine with dGMP, pdG-dC, and pdC-dG (Figure 13, supplementary material) indicate that actinomine binds more strongly to the complementary deoxydinucleotides in comparison to the deoxymononucleotide dGMP. Furthermore, actinomine appears to bind with similar affinity to either pdG-dC or pdC-dG. Since both ethidium bromide and actinomine bind strongly to the pyrimidine-purine sequence dinucleotide, pdC-dG, we would anticipate possible competitive binding of these two drugs at pyrimidine-purine (and probably other) intercalation sites on the nucleic acid double helix.

The binding data in Figures 11 and 12 may also be analyzed with the McGhee and von Hippel (1974) equations (the reader is also referred to Crothers (1968) and Zasedatelev et al. (1971)). The ethidium data shown in Figures 11 and 12 (after expressing r in terms of base pairs) may be satisfactorily represented by eq 10 of McGhee and von Hippel (1974) using an intrinsic association constant of 1.3×10^5 M^{-1} with a value of n equalling two base pairs. The value of $n = 2$ corresponds to ethidium binding to DNA at every other base pair at saturation (e.g., see Crothers, 1968). The ethidium-DNA binding experiments of Le Pecq and Paoletti (1967) yield similar results ($K = 1.6 \times 10^5$ M^{-1}) when analyzed in the same manner (J. B. Le Pecq, personal communication). Gaugian et al. (1978) have recently used the McGhee and von Hippel equations to analyze the competitive binding of two intercalating drugs to DNA. The data for the binding of ethidium to DNA in the presence of actinomine (Figure 12) may be satisfactorily represented with these competitive binding equations (Gaugian et al., 1978) by using an intrinsic association constant of 1.7×10^5 M^{-1} and a value of $n = 2$ for actinomine. On the other hand, the data for the binding of ethidium to DNA in the presence of actinomycin D (Figure 11) could not be satisfactorily represented by any theoretical binding curves calculated with these competitive binding equations. An arbitrary range of intrinsic association constants of 10^5 to 10^7 M^{-1}, and a range of $n = 4$ base pairs to $n = 6$ base pairs were used to represent the actinomycin D binding to DNA. The failure to find agreement between the calculated curves and the experimental data is consistent with the interpretation that ethidium and actinomycin D do not compete for the same binding sites at low r values (<0.12), but it must be noted that the lack of agreement between the theoretical curves and the experimental data may result from the use of McGhee and von Hippel type equations in which it is assumed that each drug binds to all the intercalation sites with the same intrinsic affinity. Since a

number of groups have shown that this assumption is not appropriate for actinomycin D binding to DNA [e.g., see the review of Meienhofer and Atherton (1977), as well as Wells and Larson (1970) and Müller and Crothers (1968)], and since ethidium also appears to exhibit a range of intrinsic binding constants for the various intercalation sequences (e.g., see the references cited in both this manuscript and in Kastrup et al., 1978), we conclude that neither the Scatchard analyses nor the usual McGhee and von Hippel analysis [eq 10 of McGhee and von Hippel (1974), or the competitive binding equations of Gaugian et al. (1978)] will provide an exact quantitative analysis of the actual binding situation. However, both approaches lead to the same *qualitative* conclusion that ethidium and actinomycin D do not appear to compete for the same binding sites at low r values (<0.12), whereas ethidium and actinomine do appear to compete for the same binding sites (Figures 11 and 12). It is this qualitative result which is important, rather than any particular emphasis on the apparent binding constants derived from either the Scatchard analysis or the McGhee and von Hippel analysis.

Discussion

Our investigations of Ethidium bromide binding to ribodinucleotides and deoxydinucleotides have been directed toward elucidating the mode of binding of ethidium bromide to nucleic acids. Experiments utilizing visible absorption, fluorescence, and ^{1}H NMR show that ethidium forms a miniature intercalated complex with pyrimidine 3′-5′ purine sequence dinucleotides (i.e., dC-dG, pdC-dG, or dT-dA compared to dG-dC, pdG-dC, or dA-dT). Studies reported here and elsewhere (Reinhardt, 1977) using visible absorption (Table I) and fluorescence spectroscopy (Figures 2 and 4), as well as previous studies employing low temperature (~0 °C) circular dichroism spectroscopy in the 300-400-nm region (Krugh et al., 1975), show that ethidium bromide in solution with dC-dG (or pdC-dG), dT-dA (or pdT-dA), or the complementary mixtures dC-dA plus dT-dG or dT-dT plus dA-dA exhibits spectral properties characteristic of the properties associated with ethidium bound to double-stranded DNA. Other dinucleotide sequences such as dG-dC, dA-dT, and the non-self-complementary deoxydinucleotides form complexes, but the visible absorbance, fluorescence, and circular dichroism spectra are not similar to the spectra characteristic of an .thidium bromide-DNA complex. *We want to emphasize* that ethidium bromide will bind to sequences other than pyrimidine-purine on DNA, RNA, and synthetic polynucleotides (Elliott, 1963; Le Pecq et al., 1964; Ward et al., 1965; Waring, 1965, 1966; Le Pecq and Paoletti, 1967, Aktipis and Martz, 1974), and thus the pyridine-purine sequence preference exhibited by ethidium bromide binding to dinucleotides must be a relative preference and *is not an absolute specificity*. The origins of the sequence-dependent binding properties of ethidium are most likely a result of the stacking forces involved between the nucleotide bases and the planar intercalated phenanthridium ring of ethidium as well as the stacking forces of the nucleotide bases (Krugh and Reinhardt, 1975; Reinhardt, 1977). The fact that ethidium exhibits sequence isomer preferences on the dinucleotide level strongly suggests that ethidium will bind to the various sequences available at intercalation sites on DNA and RNA (or other double-stranded polynucleotide systems) with significantly different binding constants (for example, see Baguley and Falkenhaug, 1978).

Proton magnetic resonance spectroscopy provides a sensitive means for determining the molecular geometries of the ethidium-deoxydinucleotide complexes. The magnetic anisotropy ring currents of the nucleotide bases result in changes in the chemical shifts of ethidium bromide protons as a consequence of complex formation (e.g., see Dwek, 1973; Emsley et al., 1965). In the NMR spectra of ethidium complexes with dC-dG [Figures 6 (supplementary material) and 7], dT-dA (Figure 9, in supplementary material), and the complementary mixture dC-dA plus dT-dG (Figure 10, in supplementary material), we observe that *all* of the protons on the phenanthridinium ring are shifted upfield by at least 0.5 ppm and that specific protons on opposite sides of the ring [for example, the H(7) and H(2) protons] are shifted upfield approximately 0.9 ppm due to complex formation. If we now consider the magnitude and radial dependence of the ring currents of the nucleotide bases (Giessner-Prettre, et al., 1976; Shulman et al., 1973), as well as the relative size of the phenanthridinium ring (Figure 1), the only geometry consistent with the NMR data involves the vertical stacking of the phenanthridinium ring between *two* nucleotide base pairs in the form of a miniature intercalated double-helical complex (i.e., a 2:1 dinucleotide-drug complex) in which the long axis of the phenanthridinium ring is roughly parallel to the base pair. This is particularly evident from the dramatic role of complementarity and the magnitudes of the indiced chemical shifts observed in the spectra of ethidium with the dC-dA plus dT-dG mixture (Figure 10, in supplementary material).

Similar observations in our NMR studies of the ethidium-ribodinucleoside monophosphate complexes led us to propose that the formation of the 2:1 nucleotide-drug intercalated complex proceeds through the reversible formation of 1:1 nucleotide-drug intermediates in a stepwise fashion (Krugh and Reinhardt, 1975). Subsequent kinetic studies by Davanloo and Crothers (1976) on the association of ethidium bromide with the complementary pyrimidine-purine sequence deoxydinucleotides indicated a rapid bimolecular reaction, which is presumably associated with the formation of the 1:1 nucleotide-drug intermediate, followed by a slower first-order step, which presumably involves a rearrangement of the 1:1 complex to accommodate a second dinucleotide to form the intercalated 2:1 nucleotide-drug complex. Only bimolecular kinetics (or extremely fast relaxations) were observed in ethidium bromide solutions with the purine-pyrimidine sequence dinucleotides (Davanloo and Crothers, 1976). The single exponential decay and fluorescence lifetime of 23 ns for the ethidium solution in the presence of excess C-G are remarkably similar to the fluorescence lifetimes associated with intercalated ethidium-DNA or ethidium-RNA complexes (Burns, 1969) and strongly suggest that under conditions of excess dinucleoside the predominant form of the solution complex is a 2:1 C-G-ethidium bromide intercalated miniature helix. It is important to recognize that under conditions where the dinucleoside is not in excess (supernatant I, Table II) several different stacked intermediate complexes may exist in equilibrium, which give rise to shorter lifetime components observed in these solutions. The two distinct fluorescence lifetimes observed in the polycrystalline ethidium bromide-C-G sample (Table II) indicate the presence of two distinct environments of the ethidium bromide molecules in the cocrystalline sample. These results are thus consistent with the X-ray crystallographic analysis of the 2:2 ethidium bromide-iodoC-G complex (Tsai et al., 1975; Jain et al., 1977) in which there were two types of ethidium. Our measurements of the lifetimes of the ethidium bromide solution complex with C-G have provided a link between the model dinucleotide solution studies and the binding of ethidium bromide to polynucleotides, while the fluorescence lifetime measurements of the crystals have provided an important link between the solution studies and the X-ray crys-

tallographic studies of Sobell and co-workers (Tsai et al., 1975, 1977; Jain et al., 1977).

The competitive-binding studies of ethidium bromide and actinomycin D (Figure 11) appear to indicate that these two intercalating drugs bind to DNA in a noncompetitive manner at low DNA-bound ethidium bromide to nucleotide phosphate ratios (i.e., $r < 0.12$, Figure 11). It is therefore quite likely that ethidium bromide and actinomycin D preferentially intercalate at different sequences along the DNA double helix. The pyr(3′–5′)pur sequence binding preferences of ethidium bromide and the opposite pur(3′–5′)pyr sequence binding preferences of actinomycin D (as predicted from the results of the model drug–dinucleotide studies) are both consistent with and suggest that the drugs do prefer to bind to different sequences. At high r values, in the presence of actinomycin D ($r > 0.12$, Figure 11), we observe a nonlinear trend in the Scatchard plot which is characteristic of a secondary competitive-type interaction between ethidium and actinomycin D. This would be observed if ethidium and actinomycin D competed for identical sites on the DNA helix. Alternatively, it is possible that ethidium bromide and actinomycin D bind noncompetitively at different sites, but because of neighbor exclusion and steric interference they *appear* to compete for identical sites as the binding of ethidium bromide approaches saturation. A combination of these effects is the most likely explanation. Blau and Bittman (1975) have reported the displacement of DNA-bound actinomycin D upon the addition of relatively high concentrations of ethidium bromide in solutions of low ionic strength. In separate control experiments, we have shown from visible absorption spectroscopic experiments that DNA-bound actinomycin D is effectively displaced *only* upon the addition of high concentrations of ethidium bromide (Reinhardt, 1977). We therefore conclude that ethidium and actinomycin D bind in a noncompetitive fashion to independent sites on the nucleic acid double helix in the linear region of the binding isotherm shown in Figure 11.

The competitive binding behavior observed for the ethidium bromide–DNA interaction in the presence of actinomine (Figure 12) provides further evidence of the specificity of the interaction of these drugs with DNA and also illustrates the effect which the groups attached to the phenoxazone ring have on the binding of the drugs. However, it should also be noted that actinomine carries a +2 charge at neutral pH, whereas actinomycin D is uncharged at neutral PH.

The preferential binding of drugs to the various nucleic acid sequences is a relatively new observation which has potential implications for, and applications to, a wide area of nucleic acid chemistry (e.g., see Müller and Crothers, 1975; Müller and Gautier, 1975; Krugh et al., 1977; Patel and Canuel, 1976; Wells and Cantor, 1977; Jones et al., 1978; and references therein). In the following paper of this issue, we present detailed optical studies on the binding of ethidium to deoxytetranucleotides as an extension of the study of the binding of ethidium bromide to deoxydinucleotides and DNA.

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Supplementary Material Available

Figures 3, 5, 6, 9, 10, and 13 showing additional spectra and visible absorption titrations (8 pages). Ordering information is given on any current masthead page.

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