Binding of Ethidium to the Nucleosome Core Particle. 2. Internal and External Binding Modes†

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ABSTRACT: We have previously reported that the binding of ethidium bromide to the nucleosome core particle results in a stepwise dissociation of the structure which involves the initial release of one copy each of H2A and H2B (McMurray & van Holde, 1986). In this report, we have examined the absorbance and fluorescence properties of intercalated and outside bound forms of ethidium bromide. From these properties, we have measured the extent of external, electrostatic binding of the dye versus internal, intercalation binding to the core particle, free from contribution by linker DNA. We have established that dissociation is induced by the *intercalation* mode of binding to DNA within the core particle DNA, and not by binding to the histones or by nonintercalative binding to DNA. The covalent binding of [^{3}H]-8-azidoethidium to the core particle clearly shows that <1.0 adduct is formed per histone octamer over a wide range of input ratios. Simultaneously, analyses of steady-state fluorescence enhancement and fluorescence lifetime data from bound ethidium complexes demonstrate extensive intercalation binding. Combined analyses from steady-state fluorescence intensity with equilibrium dialysis or fluorescence lifetime data revealed that dissociation began when ~14 ethidium molecules are bound by intercalation to each core particle and <1.0 nonintercalated ion pair was formed per core particle.

The mechanism most frequently associated with the binding of planar molecules is intercalation, the insertion of the planar portion of the molecule between the base pairs of DNA[1] (Lerman, 1961, 1963, 1964). The intercalation binding of planar dye molecules to free DNA and RNA has been directly observed by X-ray crystallography for several compounds (Fuller & Waring, 1965; Sobell et al., 1977; Jain et al., 1977, 1979; Reddy et al., 1979; Sakore et al., 1977; Quigley et al., 1980; Neidle et al., 1987). Further, intercalation has been shown to be the primary binding mode to free DNA under a range of ionic strength conditions (Jones et al., 1980; Wilson & Lopp, 1979). But do intercalating dyes bind in the same manner when DNA is wrapped around the histone octamer to form the nucleosome structure? The nucleosome differs from free DNA in its charge/mass ratio (Donecke, 1977; Mirzabekov & Rich, 1979; Girardet & Lawrence, 1979; McGhee & Felsenfeld, 1980), its average twist angle (Fasman, 1977; Simpson & Shindo; 1979), and its solubility properties (Perry & Chalkley, 1982; Komaiko & Felsenfeld, 1985; Ausio, 1986).

The structure of the nucleosome and/or the conditions used to measure dye binding to the nucleosome may enhance *nonintercalative* dye binding modes in at least three ways. First, nucleoprotein complexes are most frequently studied under low ionic strength conditions which favor external, electrostatic association of cationic dye molecules (Davidson et al., 1977a,b; Bloomfield et al., 1974). Whereas for free DNA, electrostatic binding is usually minimized through the utilization of near-physiological ionic strength conditions (Bloomfield et al., 1974), such higher ionic strength conditions destabilize the DNA/histone complex, inducing dissociation and aggregation (Yager et al., 1989 1984; Ausio et al., 1984; Cotton & Hamkalo, 1981; Stacks & Schumaker, 1979; Lilley et al., 1979). Second, it is possible that the association of histone proteins to the nucleosomal DNA may specifically inhibit intercalation binding as compared to outside binding. Finally, the histones themselves may create new binding sites for ethidium within the core particle which are absent in free DNA. It is not known if intercalators can interact with histones in the presence of DNA, possibly giving rise to spectroscopic changes similar to that of intercalation binding to DNA. Although some studies of core particle interactions with planar dye molecules have indicated that bound dye molecules do display fluorescence properties consistent with intercalation, most of these measurements were made on core particles containing extended DNA tails. Since we have demonstrated that the binding affinity is 100-fold higher to free DNA than to core particles under the same conditions (McMurray & van Holde, 1991), it is certain that dye molecules will bind to such free DNA tails before binding to core particle DNA. Thus, is not entirely clear from earlier studies whether or not intercalation of molecules into DNA can occur when that DNA is wrapped around a histone octamer.

We have prepared homogeneous, trimmed core particles which contain DNA of length 145 ± 3 bp. In studying their properties with the intercalating dye ethidium bromide, we have previously reported that the binding of ethidium to the nucleosome core particle results in a stepwise dissociation of the structure which involves the initial release of one copy each of histones H2A and H2B (McMurray & van Holde, 1986). Dissociation requires that a critical ratio of bound dye [mol of ethidium/mol (bp) of DNA], $\nu = 0.10$, be present on the core particle (McMurray & van Holde, 1991). Below the

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[1] Abbreviations: bp, base pair(s); EDTA, (ethylenedinitrilo)tetraacetic acid; DNA, deoxyribonucleic acid; MPE, methidiumpropyl-EDTA·Fe(II); DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; H2A, histone protein 2A; H2B histone protein 2B; pBr322, plasmid pBr322; Hpa II (type 2), restriction endonuclease; SDS, sodium dodecyl sulfate.

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critical value, absorbance and fluorescence measurements have indicated that binding of ethidium does occur. However, it remains to be determined if binding of ethidium in the predissociation region occurs by intercalation alone or whether alternative mechanisms such as electrostatic binding to DNA or hydrophobic interactions with the histone core are also involved. Similarly, it remains to be determined which binding mode of ethidium induces the dissociation. In this report, we examine the absorbance and fluorescence properties of intercalated and outside forms of ethidium bound to DNA, histones, core particles, and a nonintercalative polyelectrolyte, poly(vinyl sulfate). From these studies, we have determined that ethidium binding to homogeneous core particles is a specific result of intercalation binding to DNA and is not the result of nonspecific interaction with the histone proteins or electrostatic interaction with DNA.

Materials and Methods

(I) Preparation of 145 ± 3 bp Native Core Particles. Highly homogeneous chicken erythrocyte core particles were prepared as described in McMurray and van Holde (1986).

(II) Electrophoresis. (A) Native Core Particle and Denaturing Gel Electrophoresis. Native core particle gels and single-stranded denaturing gels were prepared as described in Yager and van Holde (1984).

(B) Protein Gel Electrophoresis. The protein component of the core particles was analyzed by standard SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) and stained with Coomassie blue.

(C) Laser Densitometry and Quantification of DNA. DNA or core particle bands were detected by laser scanning densitometry with a Zenieh soft-laser densitometer, Model SL-504-XL, interfaced with an Apple II-C microcomputer. The percent dissociation was quantified as described in McMurray and van Holde (1986, 1991).

(III) Sucrose Gradient Analysis. Ethidium complexes with the core particle and free DNA were analyzed on 5–20% linear sucrose gradients buffered with 10 mM Tris, pH = 8.0, and 0.1 mM EDTA as described in McMurray and van Holde (1986).

(IV) Photoaffinity Labeling of Core Particles with [^3H]-8-Azidoethidium Bromide. [^{3}H]-8-Azidoethidium bromide ([^{3}H]-8-AzEB) was synthesized by the method of Graves et al. (1977) and kindly provided by L. W. Yielding. All 8AzEB stocks were dissolved in H_2O and checked for impurities or degradation by thin-layer chromatography and absorbance spectroscopy in the 350–700-nm range. Individual core particle/8-AzEB complexes were mixed such that the final concentration of core particle DNA was 1.5×10^{-3} M bp and the input ratio of dye to bp (R) ranged from 0 to 0.4. All samples were mixed under photographic safe lights using red filters. Samples (118 μL) were equilibrated for 10 min on ice prior to photoactivation and were kept on ice during the photoactivation procedure. Maximum photoactivation was achieved by exposure to unfiltered light from a Sylvania Daylite bulb for 10 min. After photoactivation, the samples were ethanol precipitated three times to remove noncovalently bound dye. The samples were resuspended in SDS sample buffer (Laemmli, 1970), without boiling, and the protein component was analyzed on a 0.8-mm 15% polyacrylamide gel utilizing a 6% stacking gel (Laemmli, 1970). The 20-cm gels were run at 60 mA (constant current) with cooling to 21 °C. The protein bands were detected by staining with Coomassie blue as described in Laemmli (1970). All four histone bands were cut from the gel, crushed, and extracted by shaking overnight at 37 °C in 10 mL of scintillation fluid containing 10% protosol–econophor in H_2O. The level of radioactivity in each sample was detected by scintillation counting. The fraction of the 8-AzEB that formed covalent bonds was calculated by

$$\text{fraction adducts} = \frac{(\text{sample counts}) - (\text{dark control})}{\text{total counts (corrected for temperature)}} \quad (1)$$

The dark control was a sample with a dye to bp ratio identical with that of the activated sample which was not exposed to light, but otherwise went through the entire purification procedure. The number of covalent bonds formed by 8-AzEB per histone octamer was calculated by

$$\text{adducts/octamer} = (\text{fraction adducts})(R)(145) \quad (2)$$

where R is the input ratio, the number of moles of dye added per mole of DNA base pairs, and 145 is the number of base pairs per core particle.

(V) Absorbance Spectroscopy and Equilibrium Dialysis. Absorbance spectroscopy and equilibrium dialysis experiments were analyzed on a Varian 219 interfaced to an Apple II microcomputer or on a Varian 2200 spectrophotometer as described in McMurray and van Holde (1991). Absorbance measurements were converted to fraction bound, ν, and free dye as described in McMurray and van Holde (1991). The computer-calculated nonlinear least-squares best-fit values for K, n, and ω according to the McGhee and von Hippel (1974) site exclusion model were obtained as described by McMurray and van Holde (1991). Extinction coefficients were determined by absorbance measurements at the same wavelength as the free dye (λ = 480 nm).

(VI) Steady-State Fluorescence Measurements. Fluorescence intensity and anisotropy measurements were made on a computer-interfaced fluorescence spectrophotometer, originally described in Ayres et al. (1974). Excitation of ethidium was by the 546-nm line of a Hg/Xe arc isolated through two grating monochrometers and polarized vertically with a double Glan–Taylor polarizer. Emission was monitored at 585 nm and isolated by a grating monochrometer, after passage through a double Glan–Taylor prism polarizer oriented either parallel ($F_\parallel$) or perpendicular ($F_\perp$) to the excitation polarizer. Emission was detected at right angles to both the propagation and polarization directions of the exciting light. Stray excitation was removed with a Corning CS 3-66 cutoff filter (less than 1% transmission below 562 nm). A sensitivity correction (Ayres et al., 1974) was made for differential light transmission in the two polarization modes. After correction, the fluorescence intensity was calculated as $F = F_\parallel + 2F_\perp$ and the fluorescence anisotropy as $r = (F_\parallel - F_\perp)/F$. The temperature was maintained at 20 °C.

(VII) Fluorescence Lifetime Measurements. Fluorescence decay measurement were made on a monophoton decay instrument described by Small et al. (1984) with the following changes. (1) The dye laser was operated with rhodamine 575 (Exciton) in ethylene glycol (Libertini & Small, 1987). (2) The dye laser was pumped by the frequency-doubled output (532 nm) of a mode-locked Spectra Physics Series 3000 neodymium:YAG laser. (3) Interactive mode was not used. (4) Hammamatsu R1564U microchannel plate photomultipliers were used. (5) The photomultiplier output signal was sent to a Lecroy Model VV101ATS amplifier; the amplified signal was then split to provide the timing signal and the energy windowing signal (Hutchings & Small, 1990). The latter was then amplified and shaped through an Ortec 474 timeing filter amplifier using a 500-ns integration time constant (in place of the original Ortec preamplifier). (6) In some experiments, the stop signal for the time-to-amplitude converter (TAC) was

provided by the cavity dumper "sync-out" signal via an Ortec 416A gate-and-delay generator.

Excitation was 550 or 555 nm as indicated. Emission was collected through a broad-band interference filter followed by a 3-mm Corning CS 3-73 cutoff filter. This combination had a maximum transmittance of 29% centered at 593 nm and a half-width of 15 nm, tailing to the red. Temperature was maintained at 22 °C.

Analysis of the data was done on a PDP 11/34 computer (Digital Equipment Corp.) by the method of moments (Isenberg & Dyson, 1969; Small et al., 1989) using moment displacement (MD) (Isenberg, 1973; Small & Isenberg, 1976, 1977) and λ invariance testing (Isenberg & Small, 1982). The programs and a booklet describing their use are available from E. Small, Oregon State University, Corvallis, OR 97331-6503. The results were also analyzed by least-squares iterative reconvolution (LSIR) using a program developed in the lab of B. Hudson, University of Oregon, Eugene, OR. The results were essentially indistinguishable from the MD results. All data were colleced in 1024 channels at 0.1856 ns/channel. Total counts in the measured scatter profiles were generally between 7×10^6 and 13.0×10^6, and the decays usually contained 33.0×10^6 counts.

Results

The binding of ethidium to the nucleosome core particle results in a stepwise dissociation which begins with the relase of one copy each of H2A and H2B (McMurray & van Holde, 1986). In addition to intercalation binding to the DNA, ethidium may associate with the core particle through electrostatic interactions with DNA phosphates or through hydrophobic association with the histone proteins. To understand the effects of ethidium on core particles, it is necessary first to show that the primary target for ethidium binding is the DNA rather than the histones. Then we can ask whether the dissociation results from intercalation binding or from nonintercalation binding to the DNA, with consequent displacement of the histones.

(I) Binding to Protein. To estimate the extent of binding of ethidium bromide to the protein component of the core particle, two methods were employed. First, we measured the covalent binding of an ethidium derivative, [^{3}H]-8-azidoethidium bromide ([^{3}H]-8-AzEB) to the protein. [^{3}H]-8-AzEB has DNA binding properties and geometry similar to those of the parent compound (Bolton & Kearns, 1978; Graves et al., 1981; Yielding et al., 1983; Laugaa et al., 1983). Light induces decomposition of the azido group to a reactive nitrene radical which then forms a "zero-length" covalent bond with the nearest reactive group (Lwowski, 1970). Mixtures of core particles and [^{3}H]-8-AzEB at various ratios were photoactivated for 10 min with unfiltered visible light, and the core particles were ethanol precipitated to remove any noncovalently bound dye. Each sample was diluted 1:1 with SDS sample buffer (Laemmli, 1970), gently heated to 37 °C, and analyzed on 15% SDS–polyacrylamide gels. The histone bands were cut from the gel, extracted at 37 °C by shaking overnight in a scintillation mixture, and analyzed by scintillation counting. The results are shown in Figure 1. The plot of adducts formed per histone octamer versus input ratio (moles of [^{3}H]-8-AzEB/mole of bp) reveals that, at all ratios measured, little of the dye becomes covalently associated with the histone protein component. Even at the highest ratio measured the number of covalent adducts per core particle was less than 0.5. Quantitative analysis of this experiment depends, of course, on the (unknown) efficiency of the photolytic linkage of the bound ligand to protein. However, Graves et al. (1981) reported that the cross-linking efficiency of 8-AzEB is 40% to DNA. Assuming that the efficiency of cross-linking to protein is at least this high, we calculate that less than 1.0 covalent adduct is formed per histone octamer when complexed with 8-AzEB at ratios less than or equal to 0.5. We conclude from these experiments that significant binding of the dye to the histones does not occur and that the primary binding site for ethidium must be the DNA component.

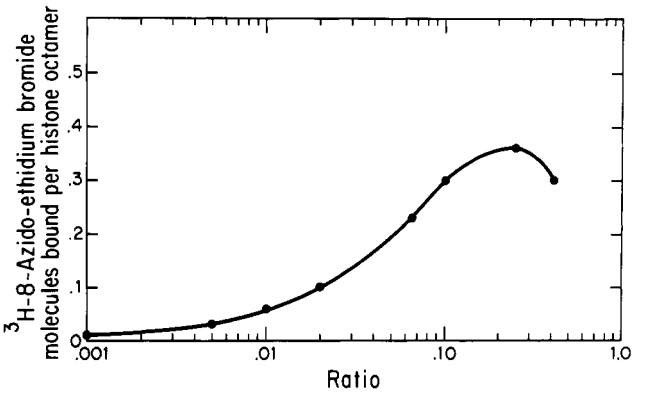

FIGURE 1: Number of [^{3}H]azidoethidium bromide molecules covalently bound per histone octamer versus log input ratio. [^{3}H]Azidoethidium was photoactivated with visible light as described in the text. The total protein component was separated by SDS–15% polyacrylamide gel electrophoresis. The labeled protein was extracted, and the amount of ^{3}H-label was detected by scintillation. The concentration of the core particle samples was 1.5×10^{-3} M bp, and the temperature was 22 °C. The buffer used in all experiments was 10 mM Tris, pH = 8.0, and 0.1 mM EDTA.

In order to provide a second estimate of the amount of dye–histone interactions, we measured absorption spectra for EB complexes with histones under two different ionic strength conditions. In 2 M NaCl and high protein concentration, the individual histone proteins will associate to form the octamer in the absence of DNA (Philip et al., 1979; Burlingame et al., 1985). Under these ionic strength conditions, electrostatic binding of EB will be abolished, but it will be possible to measure hydrophobic interactions of the dye with the folded globular region of the octamer, if such binding occurs. Lowering the ionic strength to TE buffer conditions results in dissociation of the histone octamer to a mixture of individual histone proteins and small association products (Philip et al., 1979). In TE, mostly electrostatic binding will be observable, if present. The data are summarized in Table I. The absorbance spectra, in the visible range from 650 to 350 nm, of the complexes formed between ethidium and core histones (H/EB) in TE display little to no change relative to the spectrum for free ethidium. Thus, in low ionic strength buffer, it appears that little to no ethidium binding occurs to isolated histones that are not associated to form an octamer. In the case of H/EB complexes in 2 M NaCl a small 4-nm shift was observed, but only above $R = 2.0$. For ratios at or above $R = 2.0$, two distinct isosbestic points were observed at 388 nm and at 510 nm. In 2 M NaCl at lower ratios, we observed no shifting of the spectrum and loss of the distinct isosbestic points. Thus, some weak hydrophobic association of ethidium does occur to core histones when in the form of an intact octamer, but this association only occurs to a very small extent and at very high input ratios. Hydrophobic association of ethidium with the histone octamer gives rise to distinct isosbestic points, quite similar to that observed for ethidium bound to free DNA (Le Pecq & Paoletti, 1967).

(II) Binding to DNA. (A) Absorbance Measurements. To evaluate the binding of ethidium bromide to the DNA component of the core particle at low ionic strength, we analyzed

Table I: Absorbance and Fluorescence Spectral Characteristics of Ethidium Complexed with DNA, Core Particles, Histones, and PVS at Low Ionic Strength

	DNA/EB[a]	CP/EB[b]	H/EB (2 M)[c]	TE[d]	PVS/EB[e]	EB[f]
absorbance[g]						
λ_{max} (nm)	520	520	(484)[i]	(480)	–[j]	480
$shift_{max}$ (nm)	40	40	(4)	0	30	–
ϵ_{max} (M^{-1} cm^{-1})	2300	2300	–	5750	–	5750
isosbestic point (nm)	513	517	510	–	513	–
	388	388	388	–	393	
fluorescence[h]						
λ_{max} (emission)	606	606	606	–	–	606
V	29.1	21.1	1.3	1.1	0.5–1.0	1.0
av τ_F (ns)	22.4	21.8	–	–	4.0	1.6

[a] DNA/EB = DNA/ethidium complex in TE. [b] CP/EB = core particle/ethidium complex in TE. [c] H/EB = histone/ethidium complex in TE + 2 M NaCl. The histones are composed of all four inner core histones: H2A, H2B, H3, and H4. In 2 M NaCl, the four individual histones will associate as an intact octamer. [d] H/EB = same as in footnote *c* except that the buffer is TE. In TE buffer, in the absence of DNA, the histones will dissociate into a mixture of individual histone proteins. [e] PVS/EB = poly(vinyl sulfate)/ethidium complex in TE. [f] EB = free ethidium bromide. [g] The absorbance properties of the individual complexes were obtained by titrating a concentrated solution of nucleic acid, histone, or polyanion stock into a solution of ethidium such that the volume change was very small. All absorbance measurements were corrected for dilution. Extinction coefficients were calculated from the extrapolated value to zero concentration of the extinction coefficient of an $R = 0.10$ complex for several concentrations of core particles. The temperature was maintained at 23 °C. The initial concentration of ethidium ranged from 1.0×10^{-4} to 1.6×10^{-4} M. The concentration of the core particle stock was 1.5×10^{-3} M; the concentration of the DNA stock 7.8×10^{-3} M; the PVS stock was 8 mg/mL; the histone stock was 2.2 mg/mL. The $shift_{max}$ = the difference in λ_{max} from the fully bound to the fully free ethidium. The isosbestic point(s) is(are) the wavelengths where the bound and free have the same absorbance properties. The points are identified as the points of intersection of the free dye and all ethidium complexes. [h] The excitation wavelength is 555 nm, and the emission was monitored at 593 nm. The λ_{max} is the wavelength of maximum emission intensity. The I_B/I_F is the ratio of fluorescence intensity from the fully bound form to the same concentration of free dye. τ_F is the average fluorescence lifetime for the free ethidium or ethidium complexes. [i] Parentheses indicate that the observed parameters were not obtained near a fully bound state. [j] (–) = not measured.

the absorbance properties of uncomplexed ethidium bromide and of the dye complexed with DNA, with core particles, and with poly(vinyl sulfate) (PVS) in 10 mM Tris, pH = 8.0, and 0.1 mM EDTA (TE). The inclusion of PVS as a binding substrate for ethidium bromide provided information on the spectral properties of the electrostatic binding mode of the dye alone. Since poly(vinyl sulfate) consists of monomer units having a single negative charge but no planar ring moiety, the binding of ethidium bromide to the polymer can occur only through an electrostatic association, unmixed with intercalation. The absorbance spectra in the visible range from 650 to 350 nm, of the complexes formed between ethidium and free DNA, core, particles and PVS result in a 30–40-nm hypochromic shift of the spectra for the bound complex relative to the free dye. All display distinct isosbestic points; however, only in the case of DNA and core particles could the spectrum for the fully bound dye be obtained (see Table I). The measured isosbestic points for DNA, core particle, and PVS complexes were 513/388 nm, 517/388 nm, and 513/393 nm, respectively, in good agreement with previously reported results (LePecq & Paloletti, 1967; Wu et al., 1980). If dye molecules have only one free and one bound form, the spectra for the complexes should overlap at the isosbestic point(s), where the free and bound forms display the same extinction coefficient. However, the validity of an apparent two-state model relies on the knowledge that no intermediate form has the same absorbance properties as either the bound or the free state. As shown in Table I, the complex of ethidium with free DNA, core particles, free histones (2 M NaCl), and PVS at all ratios of dye displayed distinct isosbestic points which differ only slightly in wavelength. The presence of a distinct isosbestic point for both EB/PVS and EB/H complexes suggests that electrostatic and hydrophobic bound states of ethidium give rise to similar absorbance properties as intercalation. We conclude from these studies that the absorbance properties of ethidium bromide do not distinguish among intercalation, electrostatic, or hydrophobic binding modes under our buffer conditions. Therefore, absorbance measurements, which will reliably measure total binding, can be used in conjunction with a second measurement to estimate the extent of *noninterca-lation* binding if the second type of measurement is sensitive to intercalation binding alone.

(*B*) *Fluorescence Intensity Measurements.* Large fluorescence intensity enhancement has been observed for ethidium bromide when intercalated into free DNA and into tRNA (LePecq & Paloletti, 1967; Olmstead & Kearns, 1977; Bolton & Kearns, 1978; Wu et al., 1980), and the fraction of bound dye can be calculated from fluorescence intensity measurements by

$$f_B = (I - I_F)/(V - 1)I_F \quad (3)$$

where I is the total fluorescence intensity of EB in the presence of nucleic acid, I_F is the fluorescence intensity for the same concentration of free EB, and V is the ratio of the fluorescence intensity of the fully bound dye to that of the free dye (LePecq & Paoletti, 1967). The fluorescence enhancement of ethidium upon binding DNA has been shown to be insensitive to electrostatic binding modes (LePecq & Paoletti, 1967); however, it has not been established if the hydrophobic association of a dye to globular protein results in any fluorescence enhancement. To establish the sensitivity of fluorescence intensity measurements to various binding modes, the fluorescence intensity ratio of the fully bound ethidium to free ethidium (V) was calculated for both free DNA and for core particles; the results were compared to the intensity ratio measured for the EB/PVS complexes and EB/histone complexes. For both nucleic acids, a first approximation of V was made by measuring the fluorescence intensity of ethidium at several dye to nucleic acid ratios, all of which represented a large excess of nucleic acid. The ratio at which we observed the largest fluorescence intensity enhancement was chosen to determine V for the respective binding substrate. We note that, for the core particle, the largest fluorescence intensity enhancement was observed near an input ratio (R) = 0.1. These results are consistent with cooperative binding of ethidium bromide to the core particles, reported in the preceding paper (McMurray & van Holde, 1991). The fluorescence intensity enhancement for the EB/DNA complex was largest near $R = 0.01$. The *observed* values for V were 28 for free DNA and 19.1 for the core particles. However, since the fraction of

Table II: Analysis of Ethidium Bound to Free DNA and to Core Particles Calculated from Steady-State Fluorescence Measurements and Equilibrium Dialysis Measurements[a]

DNA		core particles						
R^b	$f_{B(F)}{}^c$	R	$f_{B(F)}$	$S_I{}^d$	$f_B(ED)^e$	$S_B{}^f$	$S_B - S_I{}^g$	% D^h
0.005	0.90	0.001	0.56	0.08	0.52	0.08	0	0
0.010	0.97	0.006	0.82	0.7	0.80	0.7	0	0
0.023	0.98	0.05	0.91	7.0	0.85	6.2	0	0
0.053	0.97	0.12	0.86	13.7	0.88	14.0	0.30	2
0.09	0.97	0.18	0.79	21	0.87	22.7	1.7	4
0.15	0.96	0.25	0.80	28	-[i]	-	-	7
0.21	0.96	0.32	0.69	30	0.73	31.8	1.8	8
0.25	0.94	0.37	0.62	33	0.71	38.1	5.1	15
0.32	0.90	0.50	0.52	0.38	0.60	40.5	2.5	30
1.0	0.48	1.0	0.22	32	0.30	43.5	11.5	-

[a] The buffer used in all experiments was 10 mM Tris, pH = 8.0, and 0.1 mM EDTA. The core particle concentration was 4.0×10^{-5} M bp; the concentration of free DNA was 7.3×10^{-5} M bp. Temperature was maintained at 20 °C. [b] R = input ratio = moles of ethidium added per mole of DNA bp. [c] $F_{B(F)}$ = fraction of the added dye which was bound by the nucleic acid, as determined by steady-state fluorescence measurements by $f_B = (I - I_F)/(V - 1)I_F$. [d] S_I is the number of occupied intercalation sites on 145 bp core particle DNA. S_I is determined by steady-state fluorescence measurements = ν (145 bp), where $\nu = Rf_B$. [e] $f_B(ED)$ = fraction of added dye which is bound by nucleic acid, determined from equilibrium dialysis measurements (McMurray & van Holde, 1991). [f] S_B is the number of total bound sites by any binding mode. [g] $S_B - S_I$ is the difference in total bound sites from the number of sites bound by intercalation. This number estimates electrostatic binding sites. [h] % D is the percent dissociated DNA at the respective input ratio (McMurray & van Holde, 1991). [i] (-) indicates a parameter that was not measured.

bound dye, experimentally, is always less than 1.0, the correct V values were determined by dividing the observed V value by the calculated fraction bound. The new V value was used to recalculate the fraction bound (eq 3), and the calculation was iterated until V converged to a single value. The resulting values for V are listed in Table I. For free DNA, V was determined to be 29.1; V for the core particle was 21.1. In both cases, the fluorescence intensity of the fully bound form of the dye was >20 times the intensity of the same concentration of free dye. In contrast to the nucleic acid complexes, V for the electrostatic binding mode, gauged by the EB/PVS complex, was quenched relative to that for the free form of the dye. The V values ranged from 1.0 to 0.5, depending on the concentration of added dye. At $R = 0.01$, V was 1.0. Increasing the input ratio of dye to PVS resulted in a loss of fluorescence intensity and a decrease in V to 0.5 at $R = 0.10$. Thus, in the same input ratio range for which absorbance spectral shifts indicate binding of PVS, fluorescence measurements of pure electrostatic binding of ethidium did not give rise to any fluorescence intensity enhancement.

We compared the fluorescence properties of the EB/PVS and EB/nucleic acid complexes to those of EB/histone complexes in both low (TE) and high (2 M) ionic strengths. The complex of ethidium to the histones, at both ionic strengths, was characterized by a V value close to 1.0, similar to the results of the EB/PVS complexes (Table I). In contrast to EB/PVS complexes, fluorescence intensity enhancement of EB/histone complexes was not quenched, even at very high ratios of dye to DNA base pairs. Thus, neither electrostatic association nor hydrophobic interaction of ethidium with the histone octamer gave rise to any fluorescence intensity enhancement upon binding; in both cases V was approximately equal to 1.0 (Table I). We conclude that the fluorescence intensity increase due to ethidium binding to the core particle, insensitive to both electrostatic and hydrophobic binding modes, was a measure of intercalation binding alone.

Having established that fluorescence intensity enhancement can be used as a measure of intercalation, direct measurement of V for fully bound ethidium allows analysis of intercalated ethidium from fluorescence intensity measurements, as shown in Figure 2. For the free DNA, the fluorescence intensity of the ethidium complex increased linearly with increasing ratio of added dye below $R = 0.2$. In this range, most of the ethidium is binding by intercalation. Large fluorescence intensity increments were apparent up to ratios of 0.35, but, in this range, the intensity versus input ratio relationship was slightly less than linear, suggesting an increase in nonintercalated dye. Above $R = 0.35$, the slope of the fluorescence intensity versus ratio plot of the ethidium complex with free DNA decreased until, finally, above $R = 0.4$, quenching was observed. The fluorescence intensity of the core particle/ethidium complexes also displayed large intensity enhancement with increasing concentration of dye. Fluorescence intensity of the EB/core particle complexes was less than for EB/DNA complexes at all input ratios measured.

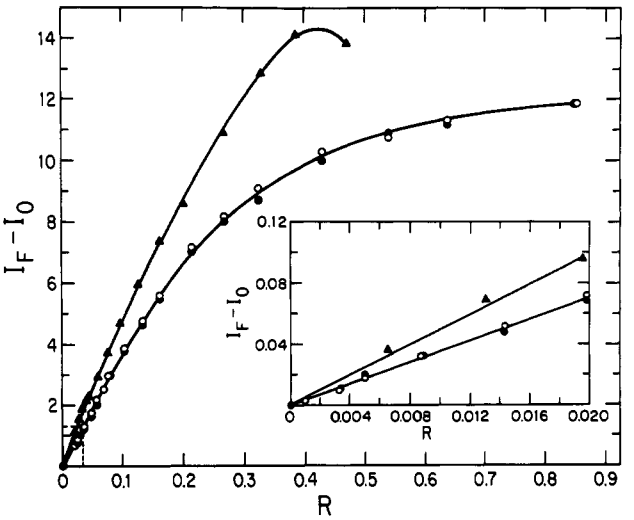

FIGURE 2: Fluorescence intensity versus input ratio for complexes of ethidium bromide with chicken erythrocyte core particles and the corresponding free DNA: (▲) free DNA; (O) core particles measured within an hour after mixing; (O) core particles measured 72 h after mixing. The excitation wavelength was 550 nm, and emission was monitored at 593 nm. I_F is the fluorescence intensity in the presence of nucleic acid, and I_0 is the fluorescence intensity of the buffer containing the same concentration of ethidium. R (input ratio) = moles of ethidium added/mole of DNA bp. The inset corresponds to the boxed region in lower left corner. The core particle concentration was 3.5×10^{-5} M bp; DNA concentration was 7.8×10^{-5} M bp. The buffer used in all experiments was 10 mM Tris, pH 8.0 and 0.1 mM EDTA. The temperature was maintained at 20 °C.

Using the V values in Table I, we calculated the intercalated fraction of added ethidium at each point in both curves, using eq 3. The results are listed in Table II. Examination of Table II reveals that the lowered overall intensity of the core particle complexes relative to free DNA at the same ratio is due to

Table III: Fluorescence Lifetime of Ethidium Complexed with Chicken Erythrocyte Core Particles and the Corresponding Free DNA

sample[a]	R[b]	τ(DNA)[c]	τ(av)[d]	% pop[e]	S_B[f]	τ(core)	τ(av)	% pop	S_B
TE (145 bp)	0.01	23.0	21.8	84.6	1.2	24.3	22.4	77.8	1.1
		15.0		9.4	0.2	17.2		20.2	0.3
		1.6		5.0		1.6		2.0	
	0.032	22.6	21.5	88.5	4.1	24.2	22.2	74.3	3.4
		15.4		11.5	0.5	17.3		21.7	1.0
		1.6		0		1.6		4.0	
	0.10	21.6	20.5	85.1	12.3	22.9	21.8	92.5	13.4
		13.9		8.9	1.3	12.2		5.5	0.8
		1.6		6.0		1.6		2.0	
	0.32	19.6	18.7	85.1	39.5	18.9	16.6	61.8	28.7
		11.0		4.9	3.7	13.6		22.2	10.3
		1.6		9.0		1.6		16.0	
	1.0	17.2	16.6	35.7	51.8	19.2	17.4	56.9	82.5
		11.2		4.3	6.3	13.8		21.1	30.5
		1.6		60.0		1.6		22.0	
HMW DNA[g]	0.032	24.5	22.4	73.8					
		17.4		22.2					
		1.6		4.0					
poly[d(A·T)][h]	0.032	25.3	24.5	90.3					
		15.2		4.7					
		1.6		5.0					
TE + 0.05 M Na^+	0.032					25.3	22.1	55.6	
						19.5		43.9	
						5.9		0.5	
						1.6		1.0	
TE + 0.15 M Na^+	0.032	23.2	21.6	48.7					
		15.7			9.3				
		1.6		42.0					
TE + 0.40 M Na^+	0.032					26.0	22.0	39.6	
						20.5		52.0	
						8.4		1.4	
						1.6		7.0	

[a] The length of DNA and the core particle was 145 ● 3 bp. For all measurements the DNA concentration was 4.5×10^{-5} M bp; the core particle concentration was 4.2×10^{-5} M bp. The temperature was maintained at 22 °C. [b] R = moles of ethidium added per mole of DNA bp. [c] τ units are nanoseconds. Lifetime data were analyzed by using a Cheng–Eisenfeld filter of 1.6 ns (the lifetime of free ethidium). [d] $\tau(\text{av}) = \sum(\%\ \text{population})(\tau_i)/\sum(\%\ \text{population})_i$; free ethidium was not included in the calculation. [e] % pop = the percentage population that displayed the fitted lifetime. The values were obtained by integrating the area below the exponential curve corresponding to the respective lifetime. [f] S_B = the number of sites bound by ligand; $S_B = (145)(R)(\%\ \text{pop})$. [g] HMW DNA is high molecular weight chicken erythrocyte DNA, MW = 1000 bp. [h] poly[d(A·T)] = alternating A·T copolymer, MW = 1000 bp.

a smaller fraction of intercalated dye. Large fluorescence intensity enhancements provide evidence that intercalation of ethidium does occur to both free DNA and highly trimmed core particles, devoid of DNA tails. Thus, we were able to estimate the amount of *nonintercalated* dye molecules from the difference between the number of bound molecules calculated from equilibrium dialysis and the number of bound molecules as measured by fluorescence intensity. While fluorescence intensity measurements were sensitive only to the number of intercalated ethidium molecules, absorbance measurements were sensitive to all bound states: intercalation, electrostatic, and hydrophobic. The level of nonintercalated ethidium molecules were compared to the population of ethidium-induced dissociated core particles (McMurray & van Holde, 1986, 1991). These results are also listed in Table II. For the core particles, the fraction of bound ethidium calculated from steady-state fluorescence measurements was quite similar to the values obtained from equilibrium dialysis measurements at all input ratios. We concluded that few non-intercalation-bound ethidium molecules were present, even at input ratios as high as $R = 0.50$. The largest variation in f_B was observed at high input ratios, as expected. The difference in the number of bound sites was calculated from both techniques and varied from 0.30 site at $R = 0.01$ to 5.0 sites at $R = 0.37$. At $R = 0.12$, the number of intercalated ethidium molecules per core particle is ~14, the average number of nonintercalated molecules is calculated to be 0.3, and a 1–2% population of the core particles is dissociated. Thus, under conditions where dissociation is beginning to occur, we calculate that less than 1 molecule of ethidium is bound per core particle by a mode other than intercalation. At $R = 0.30$, approximately 30 bp of dye are bound by intercalation, 1.8 sites are externally bound, and 8% of the core particles are dissociated. At $R = 0.3$, roughly 8 times the population of core particles is dissociated compared to the $R = 0.11$ case, yet the number of nonintercalated dye sites remains close to 1.0. We conclude that dissociation is induced by intercalated ethidium.

(*C*) *Fluorescence Lifetime Measurements.* To further test the conclusion that few ethidium molecules were bound by nonintercalation modes and that core particle dissociation was induced by the intercalation of ethidium, we measured the average fluorescence lifetime of ethidium bromide complexed to free DNA, to core particles, and to PVS. The results are listed in Tables I and III. For free DNA and for core particles, the average fluorescence lifetime of the ethidium complex is 21.8 and 22.4 ns, respectively, in good agreement with previous studies (Wahl et al., 1970; Paoletti et al., 1977; Olmsted & Kearns, 1977; Thomas & Schurr, 1983). In contrast, the fluorescence lifetime of electrostatically bound ethidium, measured in the EB/PVS complex, is about 4–5 ns (Table I). Thus, like fluorescence intensity measurements, the fluorescence lifetime of ethidium should be able to distinguish between the intercalation and nonintercalation ethidium binding modes. Additionally, the lifetime of each bound state is significantly resolved from the lifetime of free ethidium, 1.6 ns (Table I). Thus, the number of externally bound ethidium molecules can also be estimated from the difference in bound molecules calculated from steady-state fluorescence and fluorescence lifetime analyses.

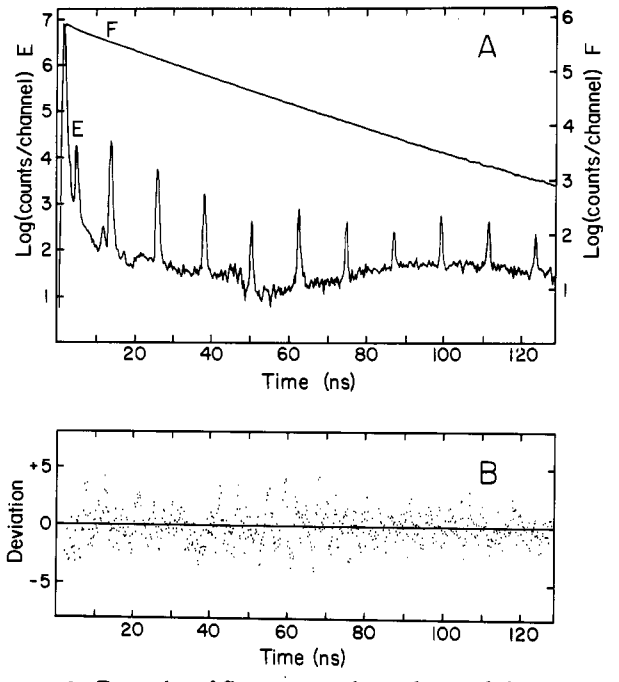

FIGURE 3: Examples of fluorescence decay data and the measured scatter profile of ethidium complexed with core particle length DNA. (A) An example of fluorescence decay data: (E) is the lamp data; (F) is the fluorescence decay data. (B) An example of the data fit.

To evaluate the *total* fraction, f_B, of bound dye molecules, we first measured the fluorescence lifetimes of ethidium over a range of input ratios for free DNA and for core particle complexes. The best fit to the decay data is obtained on the premise that, at any input ratio, some fraction of the free ethidium molecules must be present. Therefore, the lifetime of free ethidium in buffer, 1.6 ns, was fixed by use of a Cheng–Eisenfeld filter (Cheng & Eisenfeld, 1979; Small et al., 1989) in all analyses. The Cheng–Eisenfeld filter requires the computer to use the 1.6-ns lifetime in fitting the decay data. Filtering out the contribution of the free ethidium in this manner reduced the complexity of the analysis by decreasing the number of parameters. Figure 3 shows an example of the fluorescence decay data obtained; for each decay, a total of 20×10^6 data points were collected. Figure 3B shows the deviation plot for a typical analysis, demonstrating a good fit to the data. In all cases the half-width of the scatter profile was less than 0.15 ns. The data were analyzed by both method of moments (MD) and least-squares analysis in which one lifetime was kept constant at 1.6 ns. In all cases, the conclusions derived from both methods were equivalent.

The results of the fluorescence lifetime measurements are listed in Table III. For free DNA at $R = 0.01$, under our experimental conditions, the fluorescence decay curve consists of two components, in addition to the free ethidium filter. The longest lifetime component (τ_1) is 23 ns; the second component (τ_2) displays a somewhat shorter lifetime of 15 ns. The population of each component was 84.6% and 9.4%, respectively. The average fluorescence lifetime, calculated from these components, is in excellent agreement with previously reported values for single-component resolution fits to the decay curve, in each case obtained with far fewer data points (Paoletti et al., 1977; Olmsted & Kearns, 1977; Wahl et al., 1970; Thomas & Schurr, 1983). The use of the Cheng–Eisenfeld filter has little effect on the value of the average fluorescence lifetime or the fraction bound, as seen in Table IV. A multiple lifetime result appears to characterize the intercalated state of ethidium since multiple components were observed even at low ratios

Table IV: Effect of Data Collection Conditions on the Resolution of Lifetime Components of Ethidium Complexes with Free DNA and Core Particles[a]

counts	ratio	τ(DNA)	% pop	τ(av)	τ(core)	% pop	τ(av)
5×10^3	0.03	22.4	100	22.4	22.1	100	22.1
	0.10	21.9	100	21.8	22.0	100	22.0
20×10^6	0.03	22.4	100	22.4	21.9	88	22.0
					5.0	12	
	0.10	21.8	100	21.8	21.1	98	20.5
					5.0	2	
20×10^6 + filter	0.03	24.4	71	21.8	24.2	74	22.2
		17.3	25		17.3	22	
		1.6	4		1.6	4	
	0.10	22.9	88	21.6	22.9	93	21.8
		12.2	10		12.2	5	
		1.6	2		1.6	2	

[a] The length of the DNA and the core particles was 145 ± 3 bp. For all measurements the DNA concentration was 4.5×10^{-5} M bp; the core particle concentration was 4.2×10^{-5} M bp. All parameters are the same as defined in Table III.

where fluorescence intensity measurements have indicated that all the dye is bound by intercalation. Additionally, a multiple-component fit to the decay data is obtained when ethidium is complexed with high molecular weight chicken erythrocyte DNA, with poly[d(A-T)]·poly[d(A-T)] at low ionic strength, and with random sequence DNA at various ionic strengths (Table III). Thus, a multiple-component fit appears to be characteristic of ethidium bound to DNA, independent of ionic strength, base composition, or length of the DNA. Increasing the input ratio from $R = 0.01$ to $R = 0.08$ results in no significant change in the values for fluorescence lifetimes or the relative population of the individual components. However, above $R = 0.10$, we observe a decrease in the average lifetime which is characterized by a decreased τ_1, a decrease in τ_2, and a large increase in the contribution from free ethidium. The results of the fluorescence lifetime studies for the core particle over a range of input ratios are quite similar to those for free DNA. The major difference is that the average lifetime for ethidium complexed to the core particle is generally longer relative to free DNA at ratios at or below $R = 0.10$ and shorter relative to free DNA above $R = 0.1$. Additionally, the average lifetime appears resistant to decrease between $R = 0.10$ and $R = 0.20$ (data at $R = 0.2$ not shown).

From the lifetime data and the steady-state fluorescence measurements, we estimated the number of nonintercalated ethidium molecules. Making *no* assumptions about the mode of binding, we calculated the *total* fraction of bound dye by summing the populations of all components with a lifetime longer than free ethidium. This number is termed f_B. The number of bound ethidium molecules was calculated in the same manner as for steady-state fluorescence measurements and absorbance measurements, described in the preceding sections. The results are shown in Table V. The analysis reveals that, for free DNA (145 bp) under low ionic strength conditions, the number of nonintercalated ethidium molecules ranged from 1 to 15 with the maximum number observed at a high input ratio of $R = 0.5$. For the core particles, the number of nonintercalated ethidium molecules also ranges from 1 to 16, from input ratio $R = 0.006$ to $R = 0.50$. The results calculated from multicomponent fluorescence lifetime analysis were in reasonable agreement with the results shown in Table II calculated from equilibrium dialysis measurement. At $R = 0.032$, we calculate that 4–5 ethidium molecules are bound by intercalation, while 1.2 molecules are bound externally. To confirmed that roughly one ethidium molecule is bound by a nonintercalative mode, we repeated the lifetime analysis at $R = 0.032$ in [TE] + [Na^+] = 0.15 M. Under

Table V: Estimation of Ethidium Bound to Free DNA and to Core Particles Calculated from Multicomponent Fluorescence Lifetime Analysis[a]

DNA				core particles				
R^b	$f_B{}^c$	$S_B{}^d$	$S_B - S_I{}^e$	R	f_B	S_B	$S_B - S_I$	% D^f
0.010	0.86 (+)0.09	1.4	0	0.01	0.78 (+)0.20	1.4	0.7	0
0.032	0.89 (+)0.12	4.6	0.2	0.032	0.74 (+)0.22	4.4	1.2	0
0.098	0.85 (+)0.09	13.6	−0.3	0.10	0.93 (+)0.06	14.2	0	3
0.32	0.85 (+)0.06	43.2	4.5	0.32	0.62 (+)0.22	39.0	6	8
0.5	0.66 (+)0.04	50.8	14.8	0.50	0.57 (+)0.21	56.6	16.1	30
				0.032 ([NA^+] = 0.15 M)	0.45 0.13	3.6		

[a] The buffer used in all experiments was 10 mM Tris, pH = 8.0, and 0.1 mM EDTA. The core particle concentration was 4.0×10^{-5} M bp; the concentration of free DNA was 7.8×10^{-5} M bp. Temperature was maintained at 22 °C. [b] R = input ratio = moles of ethidium added per mole of DNA bp. [c] f_B = fraction of the added dye which was bound by the nucleic acid. f_B was determined by adding the best-fit populations of components from fluorescence lifetime analysis which had a lifetime longer than free ethidium (1.6 ns). [d] S_B is the number of total bound sites by any binding mode. [e] $S_B - S_I$ is the difference in total bound sites, determined from fluorescence lifetime analysis, from the number of sites bound by intercalation, determined from steady-state fluorescence measurements. This number estimates electrostatic binding sites. S_I is the number of intercalation sites on 145 bp core particle DNA. $S_I = \nu$ (145 bp), where $\nu = Rf_B$. All values for S_I were calculated from steady-state fluorescence measurements, and most values are listed in Table II. [f] % D is the percent dissociated DNA at the respective input ratio (McMurray & van Holde, 1991).

these buffer conditions, electrostatic binding of the ethidium cation to the phosphate backbone of the DNA is essentially eliminated (Table III). We observed that increasing the ionic strength of the buffer resulted in a reduction in the calculated number of bound ethidium molecules by roughly 1. At R = 0.10, we calculated that ~14 ethidium molecules are bound by intercalation, no sites are nonintercalated and 2% of the core particles have dissociated. At R = 0.32, conditions under which we detected 8–9% dissociation, roughly 33 bp of DNA are bound by intercalation and only 6 molecules associate in a nonintercalated manner. At R = 0.50, conditions under which we detected 30% dissociation, approximately 16 molecules are nonintercalated. We concluded that little external binding of ethidium bromide occurred at input ratios up to R = 0.50 and that the small fraction of nonintercalated molecules, even at high input ratios, did not account for the observed dissociation level of the DNA/histone complex.

Discussion

We have previously demonstrated that the dissociation of the core particle occurs as the result of binding ethidium above the critical level (McMurray & van Holde, 1986). The kinetics and reversibility of the ethidium-induced dissociation reaction reveal that the process is an equilibrium which is only slowly obtained (McMurray & van Holde, 1991). In this report, we have established that dissociation is induced by the *intercalation* mode of ethidium binding to DNA, rather than nonspecific binding to the histones or electrostatic binding to DNA. The covalent binding of [^{3}H]-8-azidoethidium to the core particle clearly shows that <1.0 histone adduct is formed per histone octamer over a wide range of input ratios. Under the same conditions, the large fluorescence intensity enhancement observed by steady-state fluorescence measurements and the long fluorescence lifetime of bound ethidium demonstrate that extensive intercalation binding is occurring. From these data, we conclude that ethidium binds to core particles primarily by intercalation to DNA. Combined analyses from both fluorescence intensity measurements, equilibrium dialysis, and fluorescence lifetime data reveal that only low levels of nonintercalation binding occurs at low input ratios of the dye. Under low ionic strength buffer conditions, dissociation begins when ~14 ethidium molecules are bound by intercalation and <1.0 nonintercalated ethidium is bound per core particle. We observe large increases in the population of dissociated core particles with negligible increases in the estimated number of nonintercalated molecules. Increasing the ionic strength at any ratio tested results in a decrease in the bound forms of ethidium concomitant with an increase in free ethidium. We conclude that, under buffer conditions of 10 mM Tris, pH = 8.0, and 0.1 mM EDTA, ethidium does bind primarily by intercalation to highly trimmed DNA would about a histone core, and it is this mode that induces dissociation of the histone proteins.

Registry No. Ethidium, 3546-21-2; 8-azidoethidium, 69498-50-6.

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