Fluorescence Anisotropy Decay of Ethidium Bound to Nucleosome Core Particles. 2. The Torsional Motion of the DNA Is Highly Constrained and Sensitive to pH†

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ABSTRACT: The effects of pH on the torsional flexibility of DNA bound to nucleosome core particles were investigated by using time-resolved fluorescence anisotropy decays of intercalated ethidium. The decays were collected by using time-resolved single-photon counting and were fit to a model developed by J. M. Schurr [(1984) *Chem. Phys. 84*, 71-96] with a nonlinear least-squares-fitting algorithm developed for this purpose. As the torsional flexibility of DNA is affected by the presence of an intercalating dye, the decays were studied at different ethidium bromide to core particle binding ratios. Because we see large increases in DNA flexibility and in the rotational diffusion coefficient at binding ratios of 0.6 ethidium/core particle and above, we conclude that, under these conditions, the DNA begins to detach from the protein. At lower binding ratios, we observe only small changes in the anisotropy decay. The torsional parameters obtained are a function of N, the number of base pairs of DNA between points of attachment to the histone core. Only if N is greater than 30 base pairs is the torsional rigidity of DNA on a nucleosome core particle higher than that for DNA free in solution. Also, for reasonable values of N (<30), the friction felt by the DNA on a core particle is much higher than that felt by free DNA. This indicates that the region of the DNA to which the ethidium binds is highly constrained in its motions. pH changes nearly neutrality at moderate ionic strengths (100 mM) have a substantial effect on the fluorescence anisotropy decays, particularly at early times. These analyses indicated that the observed change on increasing pH can be attributed either to a loosening of the contacts between the DNA and the histone core (increasing N) or to a substantial relaxing of the torsional rigidity of the DNA.

Intracellular pH is by no means constant throughout the cell cycle or within various organelles of the cell. A rapid rise in pH has been detected intracellularly using ^{31}P NMR following activation of eggs from *Xenopus laevis* (Webb & Nuccitelli, 1982), and just prior to maximal DNA synthesis in yeast (Gillies, 1982). There are numerous other examples [for a review, see Nuccitelli and Heiple (1982)]. More recently, Ober and Pardee (1987a,b) have shown that mitogens or growth factors activate the cellular Na/H^+ antiporter system and that such activation results in a rise of 0.25 pH unit in the intracellular pH. Accompanying this rise is the commencement of mitotic events, including DNA synthesis. These same researchers (Ober & Pardee, 1986) have suggested that the tumorigenic capacities of certain cell lines may result from an inability to regulate pH. While one could argue that the pH is affecting the optimal activity of enzymes associated with the synthesis machinery, one should still consider the possibility that the change in pH causes a conformational change in the chromatin, facilitating replication or allowing polymerases to recognize the replication origin. Recently, it has been shown that the solubility of chromatin is dependent on pH (Guo & Cole, 1989), suggesting that a rise in pH might lead to a decondensation of the chromatin prior to mitosis. It is therefore relevant to investigate whether one could observe a pH-dependent conformational transition in the primary structural unit in chromatin, the nucleosome core particle, because some control may be mediated at this fundamental level.

There have been a number of studies describing the effects of changes in the hydrogen ion concentration on the conformation of nucleosomes (Zama et al., 1978b; Gordon et al., 1979; Kawashima & Imahori, 1982; Libertini & Small, 1982, 1984; Muller et al., 1985). Among some of the biophysical techniques used to study the pH transitions are circular dichroism (Zama et al., 1978a; Libertini & Small, 1984; Muller et al., 1985), laser Raman spectroscopy (Zama et al., 1978a), the fluorescence of *N*-(3-pyrenyl)maleimide covalently bound to histone H3 (Zama et al., 1978a), sedimentation (Gordon et al., 1979; Libertini & Small, 1984), intrinsic tyrosine fluorescence (Libertini & Small, 1982, 1984), and cross-linking the histone protein core with dimethyl suberimidate (Libertini & Small, 1984). For this study, we have used time-resolved fluorescence anisotropy of ethidium intercalated in the DNA of nucleosome core particles to study a transition observed to occur centered near pH 7 and at physiologically relevant ionic strengths (Libertini & Small, 1984; Muller et al., 1985).

Ethidium is a fluorescent cation which binds to DNA by intercalation between the base pairs. By studying the rate at which initially polarized fluorescence emissions of the dye are depolarized, usually by presenting the decay as a time-dependent polarization or anisotropy function, it is possible to determine something about the environment in which the dye finds itself, as well as about the size and shape of the molecule to which the dye is attached. In the preceding paper (Brown et al., 1991), we examine the anisotropy decay at relatively long times. We find the long time data to decay monoexponentially and interpret the decay to result from the overall rotational diffusion of the particle. The correlation time that we recover (164 ± 3 ns) is consistent with what is known about the size and shape of the core particle. In this paper, we concentrate on the earlier regions of the decay which we attribute to the faster torsional motions of the DNA.

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Others have used intercalating agents to study the motions of the DNA on core particles. Genest et al. (1982) studied the decay of the fluorescence anisotropy of ethidium on core particles immobilized in 33% sucrose solutions. Ashikawa et al. (1983) used ethidium fluorescence to compare the motions of the DNA on core particles and in solubilized chromatin preparations. Wang et al. (1982) used different intercalating probes, methylene blue and tetrabromorhodamine 123, and performed triplet anisotropy measurements by monitoring the absorption anisotropy of the depleted singlet state of the dye. These earlier results are compared with ours under Discussion.

Working from an expression developed by Barkley and Zimm (1979), J. M. Schurr and co-workers have developed a theoretical expression for the anisotropy function of DNA bound to a core particle. By fitting the actual anisotropy function to this expression, one can obtain values for the torsional flexibility of DNA. In order to describe the flexing motions of the DNA, Schurr et al. proposed a model of the nucleosome core particle where a filament consisting of a number of rigid rods connected by torsional springs is constrained to girdle the equator of a sphere and is rigidly clamped at both ends. The model yields the expression for the fluorescence anisotropy decay of such a particle as a function of time (Schurr, 1984):

$$r(t) = r_0 \exp(-6D_{sph}t)\left\{\left(\frac{3}{2}\cos^2\epsilon_o - \frac{1}{2}\right)^2 + 3\cos^2\epsilon_0 \sin^2\epsilon_0 C_1(t) + \frac{3}{4}\sin^4\epsilon_0 C_2(t)\right\} \quad (1)$$

Here D_{sph} is the rotational diffusion coefficient of a particle assumed to be sphere, and ϵ_0 is 70.5°, the angle between the transition dipole and the helix axis (Schurr & Schurr, 1985). The first term in the expression describes the depolarization due to rotational diffusion. The three terms in braces describe the torsional motions in terms of correlation functions, $C_n(t)$, which are given by

$$C_n(t) = \frac{1}{N}\sum_{m=1}^{N} \exp\{-n^2\sum_{l=1}^{N} d\tilde{\gamma}_l Q_{ml}^2[1 - \exp(-t/\tau_l)]\} \quad (2)$$

with

$$Q_{ml} = [2/(N+1)]^{1/2} \sin[ml\pi/(N+1)] \quad (3)$$

$$d\tilde{\gamma} = (k_B T/\gamma)\tau_l \quad (4)$$

and

$$\tau_l = \gamma/\{4\alpha \sin^2[l\pi/2(N+1)]\} \quad (5)$$

N is the number of rigid rods between points of attachment, k_B is Boltzmann's constant, and T is the absolute temperature (Schurr, 1984). The twisting correlation functions are dependent on α, a torsional spring constant describing the properties of the torsional spring between the rigid rods, and γ, a frictional factor which is proportional to solvent viscosity. These functions predict the depolarization due to torsional twisting motions of the DNA. The depolarization which would result from bending motions of the DNA is considered negligible. The anisotropy decay depends linearly on r_0, the anisotropy at zero time. Depolarization due to dye wobble within its binding site is assumed to be fast and to contribute only to r_0.

The binding of ethidium to a core particle is known to cause dissociation of the core particle at high binding ratios (McMurray & van Holde, 1986). For this reason, the effect of the binding ratio on the anisotropy decay was investigated first. We demonstrate that if a low enough dye to core particle binding ratio is used, the results will be essentially independent of the binding ratio. Second, in this study, we show that expression 1 derived from the Schurr model provides an excellent fit to the anisotropy decay of ethidium-bound core particles though the recovered torsional rigidity and rate of rotational diffusion are both considerably higher than previously reported. Third, we demonstrate that the observed anisotropy decay of ethidium-bound nucleosome core particles is affected by changes in the hydrogen ion concentration and that these changes may be described in terms of changes in the parameters recovered by fitting the data to expression 1.

Materials and Methods

Nucleosome core particles from chicken erythrocytes were prepared as previously described (Libertini & Small, 1980). Ethidium bromide was obtained from Molecular Probes (Eugene, OR). Samples were prepared as specified for each experiment and allowed to equilibrate at least 1 h before collection to assure binding equilibrium (Small et al., 1989).

Anisotropy decay data were measured by using time-correlated photon counting with energy windowing (Hutchings & Small, 1990). The instrumentation has three basic components: the laser, which provides the source of the excitation pulse; a new fluorometer, which holds the sample, lenses, and polarizers which allow for collection of the polarized excitation or emission intensities; and the electronics, which detect the fluorescence and process the signal. The laser source, which provides picosecond pulses at 556 nm, is described in the preceding paper (Brown et al., 1991). The fluorometer has been recently constructed but is described by Winzeler (1990). A description of the electronic circuitry, including the photomultiplier tube which we use as a detector (Hamamatsu Model R1645U-06 proximity-focus double microchannel plate photomultiplier with a red-sensitive photocathode), can be found in Hutchings and Small (1990) and Small (1991). All measurements were made at 20 °C.

A complete description of the method of data collection used in this study is given in Winzeler (1990). For each measurement, two histograms of fluorescence intensity decay, denoted as $F_{\parallel}(t)$ and $F_{\perp}(t)$, were collected in which the emission polarizer was positioned parallel and perpendicular, respectively, to the vertical electric vector of the excitation source. The data collection rate was kept the same for both intensity decays and was normally 20 000 counts per second, except for some samples at very low ratios ($r < 0.01$ ethidium per core particle). Total counts collected in each decay were between 25×10^6 and 40×10^6.

Since $F_{\parallel}(t)$ and $F_{\perp}(t)$ are collected at the same counting rate, it is necessary to adjust one relative to the other by using a correction factor, S:

$$S = \frac{\text{total counts}_{\parallel}}{\text{total counts}_{\perp}} GR \quad (6)$$

Here, *total counts*$_{\parallel}$ and *total counts*$_{\perp}$ are the total number of counts contained in $F_{\parallel}(t)$ and $F_{\perp}(t)$, respectively. G and R are the ratios of the intensity of the perpendicular to the parallel components of the fluorescence emission measured for the sample, and for a solution of ethidium bromide in acetone, respectively. G corrects for the differential sensitivity of the instrument to vertically and horizontally polarized light. It is measured by using ethidium bromide in acetone, because it can be assumed that the ethidium anisotropy is zero in this nonviscous sovent. R is simply the ratio of the fluorescence intensities of the vertical and horizontal components. G and R were measured by collecting fluorescence data alternately for parallel and perpendicular components in a pulse height analyzer at a counting rate of less than 4000 counts per second,

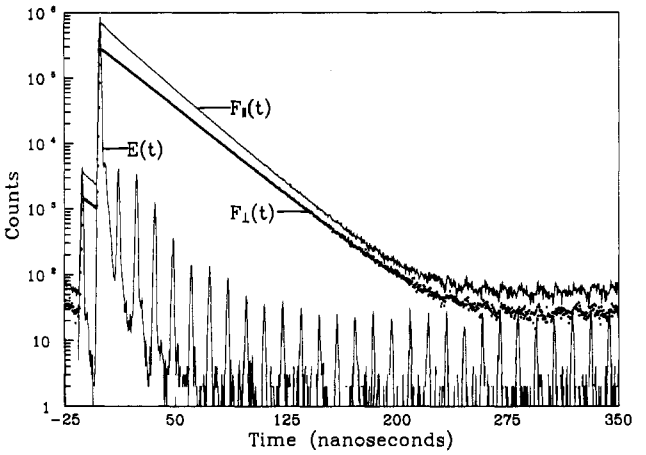

FIGURE 1: Measured functions $F_{\parallel}(t)$, $SF_{\perp}(t)$, and $E(t)$. $F_{\parallel}(t)$ and $F_{\perp}(t)$ contain 40×10^6 counts of data each. $E(t)$, the measured excitation, was collected by using a solution of dilute erythrosin in water (lifetime ~90 ps).

where we have determined the response of the instrument to be linear with incident intensity. The value of the ratios from 10 to 16 determinations was estimated by using a sine function M estimate (Andrews et al., 1972) and was used in the calculation of S.

In the most general case, the analysis objective is to fit eq 1 to the anisotropy decay which is given by

$$r(t) = \frac{f_{\parallel}(t) - f_{\perp}(t)}{f_{\parallel}(t) + 2f_{\perp}(t)} = \frac{d(t)}{s(t)} \qquad (7)$$

Here $f_{\parallel}(t)$ and $f_{\perp}(t)$ are the true fluorescence or impulse response functions which correspond to the parallel and perpendicular components of the fluorescence, respectively, and $d(t)$ and $s(t)$ are called the difference and sum functions, respectively. The impulse response function is what would be measured if one had an infinitely narrow excitation pulse and an instrument which could respond infinitely fast to the fluorescence. We will assume that the impulse response is related to the observed fluorescence response by the convolution:

$$F_{\parallel}(t) = E(t)*f_{\parallel}(t) \quad \text{and} \quad F_{\perp}(t) = [E(t)*f_{\perp}(t)]/S \qquad (8)$$

where $E(t)$ is the measured excitation (Isenberg & Small, 1982; Small et al., 1989) and S the correction factor. In some cases, to expedite the fitting process and avoid the necessity of performing a convolution routine, a numerical correction for the convolution artifact was applied. This method is described in the preceding paper (Brown et al., 1991), except that the final background correction described in that paper was not made. In this case, it is possible to solve directly for the values of the parameters which will produce the best agreement between the theoretical expression (eq 1) and the anisotropy data computed with eq 7. Use of this approach is indicated in the text. The fitting procedure, a Marquardt nonlinear least-squares analysis [see Marquardt (1963)], used in these experiments was adapted from Bevington (1969).

For most data, especially those collected over very short time ranges, the best values of the parameters α, γ, D_{sph} or r_0 were determined (without the numerical correction) by iteratively fitting the convoluted difference function (Badea & Brand, 1979).

RESULTS

Effects of Ethidium Binding on the Torsional Flexibility of DNA. A semilogarithmic plot of data, representative of those collected in these experiments, is given in Figure 1. The principal peak of the measured excitation, $E(t)$, is a clean sharp signal. Except for those near the main peak, the secondary peaks, resulting from leakage of the cavity dumper, are 4 orders of magnitude smaller than the principal peak. Though small, they become are responsible for the apparent background in the measured fluorescence at very long times. As discussed under Materials and Methods, we limit their effects either by numerically subtracting the contribution to the fluorescence made by these small excitations or by convoluting the calculated difference function with the measured excitation before fitting it to the measured difference function.

Table I: Recovered Parameters as a Function of Lower Binding Ratio[a]

R	r_0	$\alpha \times 10^{12}$ (dyn cm)	χ^2	[EtdBr] (μM)
0.0025	0.336	3.94	1.14	0.006
0.005	0.348	2.84	1.32	0.024
0.01	0.342	2.62	1.22	0.051
0.025	0.337	2.31	1.01	0.128
0.03	0.335	1.52	1.15	0.153

[a] Sodium chloride stock solution, ethidium bromide, and core particles were added, in that order, to distilled water to give a final core particle concentration of 5.2 μM and the ethidium/core particle binding ratios, R, given. The final ionic strength of the solution was 10 mM. Data were collected up to a total of 25×10^6 counts. Best-fit values of the parameters were determined by fitting the measured anisotropy to eq 1, after a numerical correction for the convolution artifact was applied to the data. The first 2 ns after the peak was ignored in the analysis to avoid inclusion of the Raman scattering peak. The value of γ was chosen to be 5.0×10^{-21} dyn cm s using global analysis. N was set equal to 15, D_{sph} was fixed at 10.0×10^5 s^{-1}, and γ was 5.0×10^{-21} dyn cm s. χ^2 reflects the goodness of fit from 2 to 170 ns.

In order to determine the flexibility of DNA on a nucleosome core particle as near as possible to its native conformation, it is important that the conformation be disturbed as little as possible by the addition of the dye. Ethidium bromide binds to DNA by intercalation between the base pairs. The effect of binding is to lengthen, stiffen, and unwind (Cantor & Schimmel, 1980) a strand of DNA. It is thus predictable that the value of the parameters recovered in our experiments would be dependent on the concentration of ethidium used and that the lower the ratio of ethidium to core particle the more closely our results will indicate the state of the DNA on the native particle. However, at a ratio of less than 0.01 ethidium per core particle, it is typically difficult to obtain an adequate count rate. Second, the most serious disadvantage is the failure of the ethidium fluorescence to mask emission by other processes. These include Raman scattering from water and low-level fluorescence of impurities in the solution. These emissions have an effect of distorting the early part (primarily less than 2 ns) of the anisotropy decay, a region which yields much information. It was our first objective in this study to determine a reasonable level where the disadvantages incurred by using a minimal amount of ethidium as a probe are minimized with respect to the advantages of using a low ratio.

Results of analyzing anisotropy data at very low binding ratios are shown in Table I. In order to obtain results that could easily be compared, we have made the simplifying assumption that $N = 15$ and that the rotational diffusion coefficient and frictional coefficient do not change with the binding ratio. The diffusion coefficient is set equal to 10.0×10^5 s^{-1} on the basis of analyses to be discussed later. The frictional coefficient was determined by a global analysis of the five datasets to be 5.0×10^{-21} dyn cm s. Because both of these values were fixed, we have forced any small differences in the curves to appear as changes in the torsional coefficient and in r_0. Such analyses result in reasonable fits to the data,

Table II: Recovered Parameters as a Function of Higher Binding Ratio[a]

R	$D_{sph} \times 10^{-5}$ (s^{-1})	r_0	$\alpha \times 10^{12}$ (dyn cm)	$\gamma \times 10^{21}$ (dyn cm s)	χ^2	[CP] (μM)	[EtdBr] (μM)
0.05	10.5	0.350	3.10	1.32	0.94	5.20	0.258
0.1	10.5	0.350	2.56	1.32	1.15	2.20	0.258
0.3	10.7	0.346	1.85	0.935	1.02	0.92	0.277
0.6	7.69	0.335	0.352	7.08	1.07	0.40	0.243
1.2	7.64	0.323	0.317	5.94	0.86	0.20	0.243

[a] Sodium chloride stock solution, ethidium bromide, and core particles (CP) were mixed to give the binding ratios, R, and final core particle concentrations given. The final ionic strength was 10 mM. Data were collected up to a total of 25×10^6 counts. Best-fit values of the parameters were determined by fitting the measured anisotropy to eq 1 after a numerical correction of the convolution artifact was applied to the decay. All parameters were allowed to vary with the exception of N which was set at 15. Time zero was considered to be the peak of the sum function. χ^2 reflects the goodness of fit from time zero to 170 ns.

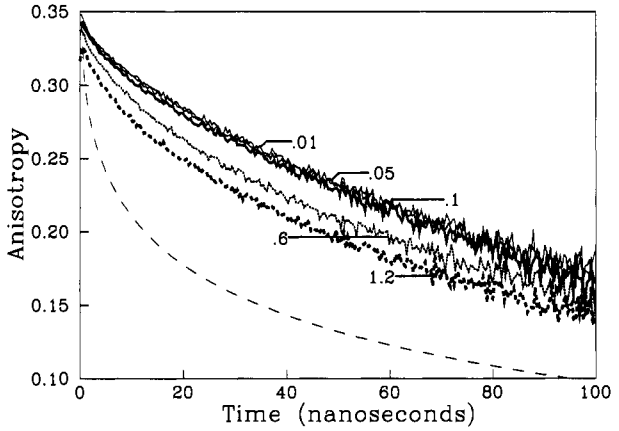

FIGURE 2: Anisotropy decays at different binding ratios. Curves are shown for five different binding ratios, sequentially decreasing as indicated in the figure. Samples were prepared as described in the footnotes to Tables I and II. Anisotropy decays were obtained from data to which a numerical correction for the convolution artifact had been applied. The data indicate that, while there are some minor changes in the shape of the anisotropy function at low binding ratios, a large increase in flexibility can be seen at binding ratios of 0.6 and 1.2, where we believe the DNA begins to detach from the core particle. The lower dashed line was generated by using the parameters describing the DNA bound to a nucleosome core particle given in Schurr and Schurr (1985). The rotational diffusion coefficient and frictional coefficient used in this projection were derived from values given in the Schurr paper but corrected for the difference in the viscosity of water between 5 and 20 °C. This curve has also been normalized to an r_0 of 0.34.

as seen in the relatively low values of χ^2, essentially constant r_0 and a value for α which varies by a factor of only 2.6 over a 12-fold change in binding ratio. Since the concentration of ethidium must be varied in these experiments, the relative amount of scatter and background fluorescence increases as the binding ratio decreases, and this increase in spurious emissions probably gives rise to the apparent change in α. Even though Figure 2 indicates little change in the shapes of the curves at very low binding ratios, the results of Table I prevent us from completely excluding the possibility that low binding ratios affect the state of the DNA.

Higher binding ratios are more easily studied, since the changes are large. Also, it is possible to maintain essentially constant ethidium concentrations while changing only the core particle concentration so that Raman scatter from the solvent remains constant and minimal. Analyses of anisotropies at five higher ratios ranging from 0.05 to 1.2 are shown in Table II. On increasing the binding ratio, D_{sph} decreases, r_0 decreases, α decreases a factor of 10, and γ decreases a factor of 6 or 7.

Note that in Tables I and II the recovered torsional coefficient is lower than the value of 3.8×10^{-12} dyn cm that was reported by Thomas et al. (1980) for free DNA, although at lower ratios we find higher α. There are a number of possible reasons for this to be true. First, we have rather arbitrarily chosen $N = 15$, based on a presumed model of the DNA on the nucleosome core particle. As will be shown below, there is an approximately linear relationship between α and N with higher N yielding higher α. We may have simply chosen too small a value of N. It is also possible that if we could extrapolate to zero binding ratio we would find α to be the same as that of free DNA. Ethidium binding to core particle DNA has been reported to be highly cooperative (McMurray, personal communication), and it is possible that multiple ethidium residues are binding near one another so that energy transfer is distorting the anisotropy decays. Such a distortion would make the DNA appear to be more flexible than it really is.

We conclude from the results in Table II that at a binding ratio of 0.6 and above the particles begin to be disrupted as evidenced by the decrease in their rate of rotational diffusion. This appears to involve the release of DNA, since the apparent flexibility also increases dramatically. Below a ratio of 0.3, there are small changes in apparent flexibility. However, we cannot at this time distinguish whether these changes are due to radiationless energy transfer between ethidium residues bound close to one another or due to real changes in DNA flexibility induced by multiple cooperative binding of the dye. If the changes shown in Tables I and II are due to energy transfer, then all of the analyses we report here will tend to *overestimate* the flexibility of the DNA.

Torsional Flexibility of Nucleosome-Bound DNA. There have been numerous studies of the flexibility of free DNA using a model similar to the one used to depict the motion of the DNA on a nucleosome core particle [see Schurr (1990) for a review]. The model for free DNA, as does the model for the nucleosome core particle, allows for variation in the torsional coefficient, α, and the frictional coefficient, γ, as well as the number of rigid rods in the flexing segment, N. Thomas et al. (1980) have fit the expression for the predicted anisotropy derived from the model for DNA to the anisotropy decays of free ethidium-bound DNA and have determined the value of the torsional coefficient to be 3.8×10^{-12} dyn cm. It is more difficult to assign a value to the torsional coefficient of DNA on a nucleosome core particle.

The recovered values of the parameters α and γ are dependent on the choice of the number of base pairs, N, between points of attachment to the core particle. This dependence is predictable and can easily be demonstrated. Once best-fit values α and γ are calculated for one N, it is possible to calculate what the best-fit value would be for any other N (Schurr & Schurr, 1985). This phenomenon is illustrated in Figure 3. For this analysis, a search was made for the best-fit values of α, γ, and D_{sph} as a function of N. The form of this function (solid line) is similar to that (dashed line) which would be expected if α and γ were determined algebraically. The values of χ^2 provided little assistance in our efforts to assign N a value. χ^2 was only marginally lower at smaller values of

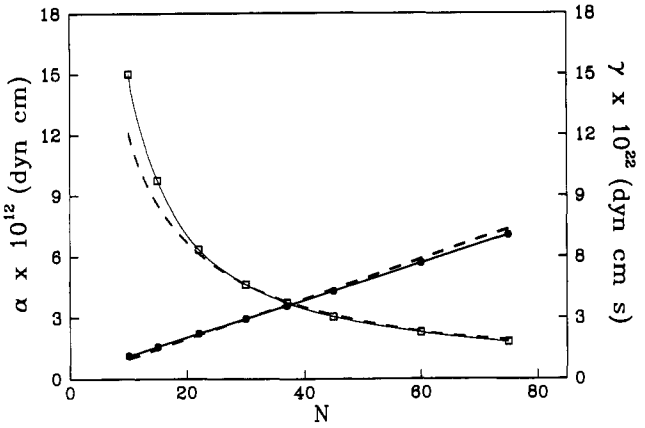

FIGURE 3: Torsional and frictional coefficients as a function of N. Solid lines show the best-fit values of α and γ for different N as obtained by iteratively fitting the difference function. α (solid circles) is projected on the left axis and γ (open squares) on the right. Dashed lines show the values of α and γ predicted from the expressions $(N + 1) = (1.02 \times 10^{13})\alpha$, and $\alpha = (1.53 \times 10^{-33})/\gamma$. The constants in these expressions were derived at $N = 15$. The sample was prepared as described in the footnotes to Table III. The data indicate that one can predict the value of either of these parameters for any value of N with some certainty.

N (e.g., 1.145 at $N = 15$ versus 1.146 at $N = 45$). Of course, the linearity of α with respect to N and the dependence of γ and α allow us to extrapolate these values to any value of N. Larger values of N were not presented in the chart because of the excessive time required to fit a dataset, not short even for $N = 15$, and increasing in proportion to the square of N.

From their analyses of the triplet anisotropy decay data of Wang et al. (1982), Schurr and Schurr (1985) report the torsional and frictional coefficients for DNA bound to a core particle to be 1.5×10^{-12} dyn cm and 9.34×10^{-23} dyn cm s, respectively. To obtain these results, N was set at 146 base pairs, and the rotational diffusion coefficient was assumed to be 6.12×10^5 s^{-1} (adjusted to 20 °C). Such a diffusion coefficient, however, implies a hydrodynamic radius of the equivalent sphere to be 62.9 Å. This size is inconsistent with studies from our laboratory [presented in Brown et al. (1991)] which have estimated the hydrodynamic radius of the equivalent sphere to be 54 Å, corresponding to a rotational diffusion coefficient of 10.2×10^5 s^{-1}. In order to determine the value of this parameter independently, we have allowed it to vary along with α and γ. Additionally, because it is unlikely that DNA is clamped to the nucleosome only at its ends, we have primarily considered values of N which are much lower than 146. For $N = 15$, at a binding ratio of 0.03, it is possible to obtain a very good fit to the data as shown in Figure 4. Here α is approximately 1.5×10^{-12} dyn cm, and γ is about 8×10^{-22} dyn cm s (see Table III). While the value of α is identical with the value reported by Schurr and Schurr (1985), there is an important difference. As a result of the choice of N used (146 versus 15) in the Schurr study, 1.5×10^{-12} dyn cm was given as the maximum value of α while here it is close to the minimum.

This indicates that only if the flexing segment of DNA is shorter than 35 base pairs can one say, as has been proposed, that the DNA on a nucleosome core particle is more flexible than DNA free in solution. Over a range of values of N (from 25 to 60), α is approximately the same as the DNA free in solution. The frictional factor, even at the unlikely $N = 80$, is at least 2-fold higher than that proposed for free DNA (7.2×10^{-23} cm s at 20 °C).

Our analyses of the data indicate that the rotational diffusion coefficient of the native core particles is about 10 × 10^5 s^{-1}. This agrees within experimental error with the value reported in Brown et al. (1991). In that work, the value was determined by using a different method on data which were optimized for that purpose. Using 6.12×10^5 s^{-1} for our analysis gives a poor fit to the data at very short time (see

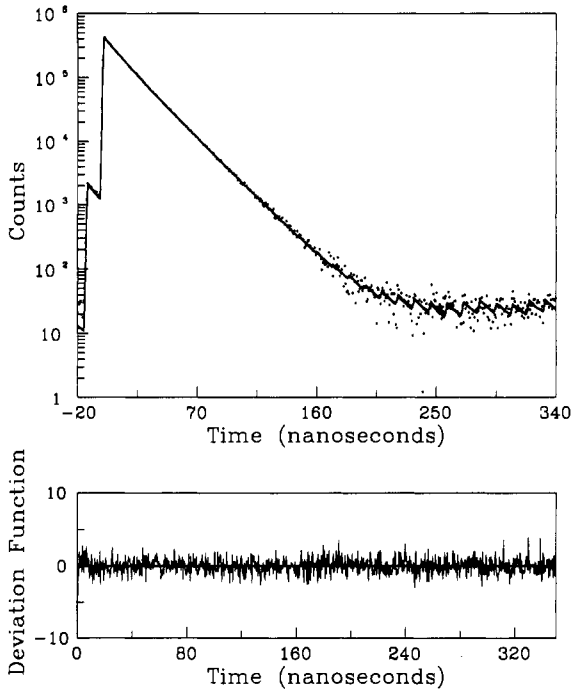

FIGURE 4: Measured difference function. The calculated curve (solid line) is shown superimposed on an example of a measured difference function, $F_{\parallel}(t) - SF_{\perp}(t)$. Also shown is the resulting deviation function which indicates the goodness of fit. Sample was prepared as described in the footnotes to Table III. For this fit, $\alpha = 1.58 \times 10^{-12}$ dyn cm, $\gamma = 9.5 \times 10^{-22}$ dyn cm s, $D_{sph} = 10.1 \times 10^5$ s^{-1}, $N = 15$, and $\chi^2 = 1.11$.

Table III: Results from Different Analyses of Four Datasets[a]

data no.	ns/channel	$\alpha \times 10^{12}$ (dyn cm)	$\gamma \times 10^{21}$ (dyn cm s)	$D_{sph} \times 10^{-5}$ (s^{-1})	χ^2
1A	0.3764	1.58	0.95	10.1	1.11
1A	0.3764	1.84	1.66	9.91	0.82[b]
1A	0.3764	0.318	6.77	6.12[c]	1.60
2A	0.3764	1.77	0.94	10.6	1.27
2A	0.3764	1.83	2.17	9.99	0.95[b]
1B	0.0928	1.93	0.73	11.2	1.67
1B	0.0928	1.76	0.86	10.6	1.70[d]
2B	0.0928	1.59	0.30	11.7	1.85
2B	0.0928	1.41	0.61	10.1	1.98[d]
SS[e]		0.164	1.17	6.12	

[a] Two datasets were collected on 2 different days using two different time ranges: 0.3764 and 0.0928 ns/channel. Samples consisted of 10 mM NaCl, 0.154 μM ethidium bromide, and 5.125 μM core particles in glass-distilled water. This gave an ethidium/core particle ratio of 0.03. Approximately 40×10^6 counts were collected for $F_{\parallel}(t)$ and $F_{\perp}(t)$ in each case. A solution of dilute erythrosin in water was used to collect the measured excitation, $E(t)$. Datasets 1A and 1B, and 2A and 2B were collected from the same sample, respectively, but using the indicated channel widths. Except where indicated, all fits were obtained by fitting the data to the convoluted difference function. N was held constant at 15. Values of χ^2 indicate the quality of fit up to 200 ns for 0.376 ns/channel and up to 85 ns for 0.093 ns/channel. [b] χ^2 was determined by fitting the anisotropy data obtained after a numerical correction for the convolution artifact was applied directly to $r_c(t)$. [c] D_{sph} was held constant at a value corresponding to a sphere with a hydrodynamic radius of 62.9 Å as proposed by Schurr and Schurr (1985) from their analysis of the methylene blue data of Wang et al. (1985). [d] D_{sph} was held constant at a value derived from analysis of data collected at 0.3764 ns/channel. This was done under the presumption that this parameter could not be resolved properly in an 85-ns time span. [e] Results reported by Schurr and Schurr (1985).

Table IV: Best-Fit Parameters for Different pHs[a]

pH	cation concn (mM)	$\alpha \times 10^{12}$ (dyn cm)	$\gamma \times 10^{21}$ (dyn cm s)	r_0	$D_{sph} \times 10^{-5}$ (s^{-1})	χ^{2b}
4.79	100	2.10	2.42	0.352	8.26	1.17
5.49	100	2.69	1.92	0.352	9.51	0.97
5.99	100	2.57	1.79	0.351	9.27	0.94
6.40	100	2.29	1.30	0.350	9.58	1.09
6.88	100	1.95	1.04	0.352	9.76	1.16
7.64	100	1.48	1.48	0.350	8.99	1.04
8.31	100	1.37	1.55	0.346	9.55	1.06
8.80	100	1.34	1.23	0.360	9.19	1.13
9.26	100	1.35	0.89	0.362	9.58	1.12
4.85	10	1.71	3.43	0.351	8.47	1.22
5.48	10	1.96	1.55	0.355	10.0	1.39
5.99	10	2.15	0.91	0.353	10.5	1.40
6.85	10	1.53	1.27	0.351	9.94	1.45
7.50	10	1.37	1.30	0.358	9.60	1.30
8.30	10	1.19	1.69	0.355	8.97	1.26
8.57	10	1.19	1.27	0.356	8.98	1.30

[a] Values were obtained by fitting the difference function using an iterative deconvolution routine. Binding ratios were 0.05 and 0.03 for the 10 and 100 mM data, respectively. For 100 mM samples, stock solution, ethidium bromide, and core particles were sequentially added to distilled water to give a final core particle concentration of 5.13 μM core particles and an ethidium/core particle ratio of 0.03. For measurements at pH <5, sodium acetate buffer was used; for 5 < pH < 7, MES buffer was used; for pH 7, the buffer was MOPS; and for pH >7, the buffer used was Tris-HCl. Solutions had a final ionic strength of 100 mM. pH was determined following the measurements. 10 mM samples were prepared by adding sodium chloride stock solution, ethidium bromide stock solution, and core particles to glass-distilled water to give a final core particle concentration of 5.12 μM. A total of 40×10^6 and 25×10^6 counts were collected for $F_{\parallel}(t)$ and $F_{\perp}(t)$ for the 100 and 10 mM samples, respectively. N was held constant at 15. [b] χ^2 describes the fit obtained beginning at the peak of the sum file out to 200 ns.

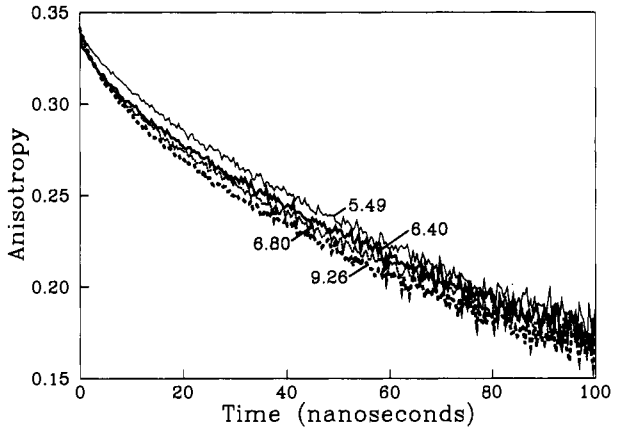

FIGURE 5: Anisotropy decays as a function of pH (100 mM salt). From top to bottom, decays are shown for pH values of 5.49, 6.40, 6.80, and 9.26 as indicated on the plot. Samples were prepared as described in the footnotes to Table IV. Anisotropy decays were obtained from data to which a numerical correction for the convolution artifact had been applied.

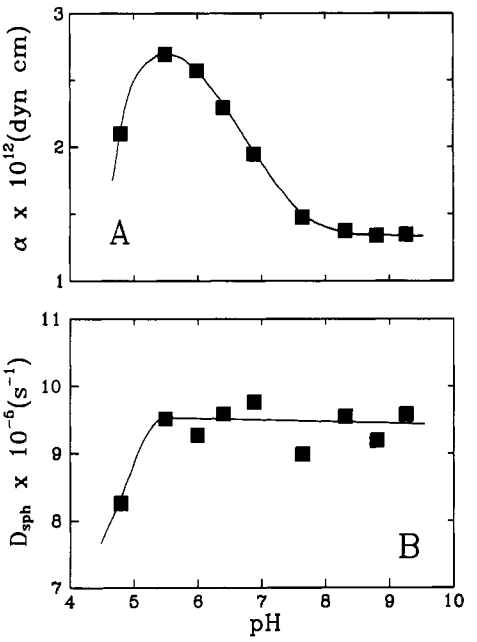

FIGURE 6: Torsional coefficient and rotational diffusion coefficient as a function of pH. Samples were prepared and collected as described in the caption of Figure 5. For (A), values of α were obtained by iteratively fitting the difference function. All other parameters, including r_0, D_{sph}, and γ, were considered as variable. For (B), values of D_{sph} were obtained by iteratively fitting the difference function. All other parameters, including r_0, α, and γ, were considered as variable.

Table III). As can be seen in Figure 4, we are able to achieve an excellent fit at every region of the decay using 10×10^5 s^{-1}.

To show the dramatic difference in apparent DNA rigidity between our ethidium results and the methylene blue triplet decay measured by Wang et al. (1982), a curve was calculated by using the decay parameters reported by Schurr and Schurr (1985) included in Figure 2. At early times, the methylene blue data decay off much faster than even the ethidium anisotropies of particles disrupted by high ethidium binding ratios.

Effects of pH on the DNA of a Nucleosome Core Particle. Analyses of anisotropy decays at different pH values in the range of about 5–9 are shown in Table IV for ethidium-bound core particles at both 10 and 100 mM ionic strength. Distinct transitions are seen just below pH 7 in the recovered values of α. D_{sph} appears to remain approximately constant as a function of pH, although there may be some decrease in this parameter evident in the 10 mM ionic strength data.

To illustrate visually the magnitude of the changes, anisotropy decays for five different pH values are plotted in Figure 5 for the 100 mM data. Note that increasing pH leads to greater depolarization of fluorescence and therefore indicates increased flexibility of the DNA. A plot of the recovered α for 100 mM data is shown in Figure 6A. Except at the lowest pH value, where we believe that the core particles are beginning to aggregate (Libertini & Small, 1984), a smooth transition is seen centered slightly below pH 7. We therefore believe that this change is related to the transition reported earlier in the steady-state intrinsic tyrosine anisotropy (Libertini & Small, 1984). Recovered values of D_{sph} are plotted vs. pH in Figure 6B. Except at the lowest pH, there is no measurable change in D_{sph}, indicating that the particles are

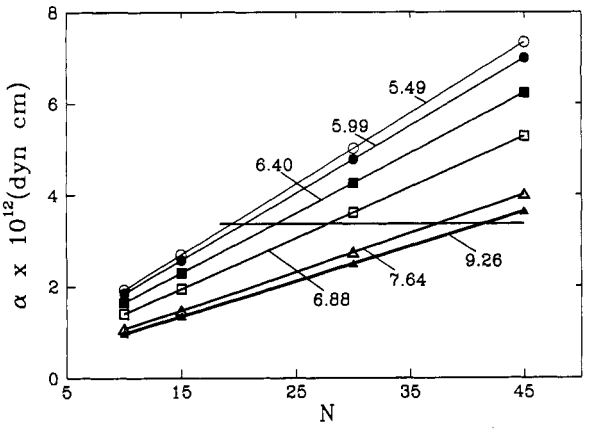

FIGURE 7: Best fit of the torsional coefficient for different N and pH values. The solid lines show the best-fit values of α for different N as obtained by iteratively fitting the difference function of pH data. The solid horizontal line at approximately the value of free DNA is included to emphasize that the observed change in α may actually be due to a change in N.

not significantly changing in size or shape on increasing pH. This agrees with earlier sedimentation results (Libertini & Small, 1984), although D_{sph} should be much more sensitive to changes in shape than the sedimentation coefficient (Small et al., 1990b). The lack of change in D_{sph} means that pH transition is not causing detachment of the ends of the DNA or we believe that we would see the kind of changes seen in Table II (an increase in flexibility accompanied by a drop in D_{sph}).

While it is also unclear as to whether there is any transition with respect to γ, it is readily apparent from the data that there is a decrease in α with pH if N is held constant. However, it is equally possible that N has changed and the torsional coefficient has remained the same. Just as was demonstrated above, it is possible to show that α increases linearly with increasing N (see Figure 7). Here a line has been drawn at $\alpha = 3.5 \times 10^{-12}$ dyn cm s to illustrate that it is possible to hold this value constant and achieve essentially the same fits by only changes N. Therefore, an acceptable explanation for the observed increase in flexibility with pH is that *the flexing segment length increases as the conditions become more alkaline*. To be responsible for the observed changes, the flexing length would need to more than double.

Discussion

By using the equations developed by Schurr et al., we have assumed that nucleosomal DNA can be modeled as a series of rigid rods connected to one another by Hookean springs and that the rods are constrained to girdle a sphere and are bound to this sphere at both ends. The recovered values of α, γ, and N are only defined in terms of the model. Other models are possible [see, for example, Chen (1990)]; however, we have attained an excellent fit to the data both at short and at long times using this model and are thus satisfied with it. Given our success with the Schurr model, we have sought to fully explore its potential and determine what it is able to tell us about the physical constants or conformation of DNA bound to a nucleosome, as well as if it can be used as an indicator of the effects of ethidium binding and hydrogen ion concentration on the DNA. There are, however, two questions which first must be answered.

The first is whether ethidium will bind to nucleosomes at low enough ratios so that the native conformation of the core particle is not significantly affected. It has been reported (Erard et al., 1979; McMurray, personal communication) that the binding of ethidium bromide to the nucleosome core particles in the absence of any linker DNA and at low ionic strengths may be a highly cooperative process. If this cooperativity holds at very low binding ratios, then a significant fraction of the nucleosomes will have more than one ethidium bound to them, even at low ratios. McMurray and van Holde (personal communication) have fit the data from spectrophotometric measurements of the concentrations of bound and unbound ethidium to the theoretical expressions of McGhee and von Hippel (1974) and have obtained values of 4.5 140, and 2.4×10^4 M^{-1}, respectively, for the number of base pairs excluded by the dye, the cooperativity parameter, ω, and the binding constant, K_a, by using this conditional probability model.

Cooperative binding implies that the binding of the first ethidium disrupts the structure to facilitate the binding of the next dye residue. Thus, the anisotropy of the ethidium is less likely to probe the state of the native core particle. Also, cooperativity implies that the binding of the second residue occurs near the first. If two ethidiums are close to one another, then it is likely that radiationless energy transfer will occur between them. If such transfer occurs at a rate comparable to the excited-state lifetime, then depolarization of the fluorescence will occur. The anisotropy will be distorted by this process and appear to decay too rapidly. Also, if enough ethidium is bound to cause dissociation, the recovered rotational diffusion coefficient would be decreased, reflecting the drastic change in the geometry of some of the molecules. These predictions are substantiated by the results given in Table II, where a large increase in the rotational diffusion coefficient and an increase in flexibility are observed at binding ratios greater than 0.3 ethidium per core particle.

Genest et al. (1982) have made similar observations about the effect of binding ratio on the fundamental anisotropy, r_0. The lowest binding ratio they reported was 0.073 ethidium per core particle. Using measurements of the steady-state anisotropy as well as measurements of the fluorescence anisotropy decays, they were able to show substantial changes in the recovered lifetimes as well as decreases in r_0 and the steady-state anisotropy between the binding ratios of 0.073 and 1.46. These researchers attribute these phenomena to fast excitation energy transfer between adjacent ethidiums. If such an observation is correct, it would suggest that if energy transfer occurs when there is on the average less than 0.1 ethidium per core particle then there is a higher than random probability that binding is occurring at adjacent sites and is therefore cooperative. Genest and Wahl (1981) have proposed that in the native core particle only a short segment of the DNA is accessible to the ethidium molecules and that the binding of a few ethidiums to this segment induces the accessibility of another segment such that the binding of eight or nine ethidium molecules is sufficient to make 93% of the DNA accessible to binding.

At relatively high ethidium ratios, we see the expected increase in DNA flexibility and decrease in diffusion coefficient (see Figure 2 and Table II). However, *as a result of our measurements, we have found that the conformation of the core particle is not significantly affected by ethidium binding when ratios of 0.3 or less ethidium per core particle are used.* Of course, it must be kept in mind that, at even the lowest binding ratios, we are probing the state of the DNA only near where the ethidium binds and only after the DNA has been altered by the binding.

The second question is the choice of N, the number of base pairs between points of attachment. It is more likely than not

that the DNA is attached to the nucleosome core particle at a number of places between the two ends. DNase I digestion of nucleosome core particles has been shown to be inhibited at sites approximately 30, 60, 80, and 110 base pairs from the ends of the DNA (Lutter, 1978), suggesting possible contacts with the DNA at these points. Thermal denaturation studies (McMurray et al., 1985) have indicated that DNA on a core particle melts from the ends inward and is clearly biphasic. These studies suggest attachment points at 20–25 base pairs from either end. Further evidence that N is much less than 146 base pairs can be found in some recent work by McMurray and van Holde (personal communication). These workers examined the binding of Fe(II)-bound methidium-propyl-EDTA (MPE) to core particles. This molecule has a methylated ethidium and is presumed to bind to the same region of nucleosomal DNA that is available to free ethidium. Upon addition of a sulfhydryl reagent such as dithiothreitol, the core particle bound MPE cleaves a strand of the DNA near the binding site. Subsequent cleavage with S1 nuclease and removal of protein produce double-stranded DNA fragments which are then run out on a gel. McMurray and van Holde find that the MPE binds only near the ends of the DNA, giving rise to fragments of an average length of about 135 and 120 base pairs. They conclude that the MPE can bind within the first 20 to 25 base pairs of DNA to either end of the DNA. We suspect that this is the most mobile segment of DNA on the particle.

Given this information, it is unlikely that N is as large as 145, and we have therefore primarily considered much smaller values of this parameter in our analyses. However, we are able to obtain equally good fits for any number of values of N (though χ^2 is marginally better for lower N; see Results for a discussion of this subject). If the number of base pairs between points of attachment is any number greater than about 30, which is a possibility, then the Schurr model implies that the torsional rigidity of the DNA on a core particle is higher than or equal to that of free DNA.

One must also consider the possibility that N is less than 30 and that the torsional rigidity of nucleosomal DNA is less than that of free DNA. Intuitively, this does not seem likely because the DNA is obviously subject to a number of constraints in its association with the core particle. Second, the number of base pairs per turn for DNA on a nucleosome core particle is thought to be 10.0, in contrast with 10.5 for free DNA (van Holde, 1988). This implies that the DNA on a core particle is more tightly wound than free DNA and suggests that the torsional flexibility of core particle DNA should be impeded, and not encouraged. However, given the similarity between the number we attain for the torsional coefficient and the one observed for free DNA, it is perhaps most plausible to assume that the torsional rigidity of DNA on a nucleosome core particle is nearly the same as free DNA. This implies that if the Schurr model is appropriate, ethidium binds to DNA on a strand which is about 30 base pairs long and which is bound at either end.

While there is some question as to what is the correct choice of N, the value of γ with respect to that of free DNA is much easier to determine. It is clear from our studies that it must be considerably higher than that corresponding to free DNA. Furthermore, there is little uncertainty about the value of the rotational diffusion coefficient, D_{sph}. The lowest point in the χ^2 surface is approximately 10×10^5 s^{-1} regardless of the choice of N. We are unable to fit the data as well using a lower value of this parameter. This would suggest that the values we recover are accurate.

Recent studies have detected transitions around neutrality (Libertini & Small, 1982, 1984; Muller et al., 1985) as well as at pH 4 (Libertini & Small, 1982). The transition observed by Libertini and Small (1982) at pH 4 was shown to result from precipitation using light-scattering measurements at 400 nm. Libertini and Small (1984) detected small changes in the CD at 284 nm centered at about pH 7 using core particles at 100 mM ionic strength, but were unable to see any significant changes when using 260 nm, where conformational changes in the histones might have been detected. Using circular dichroism, Muller et al. (1985) also demonstrated changes in the ellipticity of DNA at 283.5 nm centered at pH 6.65 using a 20 mM phosphate buffer. Libertini and Small (1984) also detected a small change in the intrinsic tyrosine steady-state fluorescence anisotropy centered around pH 7, and were able to show that this transition was blocked by cross-linking the protein core with dimethyl suberimidate. Very little change in the sedimentation coefficient was observed at $5 < \text{pH} < 8$.

The results presented here confirm that there is some change in the conformation of the core particle centered near neutrality. The earlier tyrosine anisotropy and CD results (Libertini & Small, 1984) indicated very small changes. The changes reported here on DNA flexibility are relatively large changes. Either the torsional rigidity is decreasing by a factor of 2, or the length of the flexing segment is increasing by greater that a factor of 2 (see Figures 6A and 7).

Previous workers who have used intercalating probes have come to differing conclusions regarding the torsional rigidity of the DNA on the core particle. Genest et al. (1982) used ethidium anisotropy to characterize the DNA on particles immobilized in 33% sucrose solutions. They analyzed their data as a sum of two exponentials and concluded, as we do here, that the segment in which ethidium binds is restricted in length. Ashikawa et al. (1983) also used ethidium fluorescence to examine the DNA on core particles and chromatin preparations. For core particles, they conclude, in agreement with our results, that the DNA has torsional rigidity similar to that of free DNA. Although Ashikawa et al. (1983) observe more torsional motion than is reported here, it should be noted that the single pH that they used (7.5) is above the midpoint of the loosening transition.

Wang et al. (1982) used two different DNA probes, tetrabromorhodamine 123 and methylene blue, both of which are assumed to bind by intercalation, as well as a different experimental technique. They used the polarized absorbance of the first excited singlet state to monitor the anisotropy decay of the long-lived triplet. From their results, they conclude that the DNA is much more flexible than we are reporting here and that the particle undergoes significantly slower rotational diffusion. The same methylene blue curve of Wang et al. was subsequently reanalyzed using the Schurr model by Schurr and Schurr (1985), who report values of $\alpha = 1.5 \times 10^{-12}$ and $\gamma = 7.21 \times 10^{-23}$ with $N = 145$ when corrected to 20 °C. A quick examination of Figure 3 shows that this result implies a very much greater flexibility in the DNA. A visual comparison between this result and our data can also be seen in Figure 2. Although methylene blue has been reported to bind near the proposed kinks in the DNA (Hogan et al., 1987), ethidium, as discussed above, probably binds near the DNA ends. However, the most obvious difference between our experiment and that of Wang et al. was the dye/core particle ratio (0.03 versus 2.0) used in the preparation of the samples. While the binding properties of ethidium and methylene blue may not be comparable, we have, nevertheless, found signif-

icant distortion of the core particle at ethidium per core particle ratios as low as 0.6 (see Table II).

Another possible source of discrepancy between the different results may be method of core particle preparation. Both Ashikawa et al. and Wang et al. used core particles that were leached from nuclei after treatment with nucleases. Such particles might be expected to be enriched in active sequences and contaminated by some particles containing extra DNA. Our particles, on the other hand, have extremely homogeneous DNA length because they are isolated from fragmented chromatin stripped of histones H1 and H5, and the non-histone chromosomal proteins by treatment with CM-Sephadex. They are also prepared in very high yield from isolated chromatin and therefore represent a more average particle (Libertini & Small, 1980). However, the possibility that particles from different sources might have different DNA rigidity suggests important new avenues of research.

CONCLUSIONS

The fluorescence anisotropy of ethidium intercalated into the DNA of core particles decays due to the motions experienced by the chromophore during the lifetime of its excited state. At relatively short times below ~100 ns, most of the observed decay results from the torsional flexing of the DNA; at longer times, rotational diffusion of the entire particle dominates. This paper concentrates on the flexing motions. In contrast to previous reports, we find the flexing of the DNA to be highly constrained.

We have analyzed our results in terms of the model of DNA motions developed primarily by Schurr and co-workers. This model consists of a string of cylinders connected by Hookean springs, constrained to the surface of a sphere and firmly attached at both ends. The model parameters which we seek are α (the torsional spring constant), γ (the frictional coefficient of an individual cylinder) (assumed to be a base pair), D_{sph} (the rotational diffusion coefficient), and N (the number of base pairs between the attachment points). An additional parameter, r_0 (the anisotropy at zero time), does not have direct meaning in terms of the model, but must be determined as well. This additional parameter adds considerable complexity to the data analysis. The anisotropy data do not permit us to simultaneously determine all of the above parameters. However, if we make some assumptions about the behavior of the DNA, then we must reach certain conclusions.

For example, if we assume that the torsional rigidity of the DNA is not much different than that of free DNA, then N must be much less than 146 bp, perhaps in the range of 15–30 base pairs. If this is true, then our analyses show that γ must be much higher than that experienced by free DNA. This might result from interactions between the DNA and the protein core; alternatively, the DNA motions may be impeded by immobilized solvent.

The torsional flexibility of the DNA at the point in which the ethidium binds is very sensitive to pH near neutrality at moderate (physiologically relevant) ionic strengths. Although we cannot at this time distinguish the two possibilities, our data show that, on increasing pH, either the torsional flexibility is increasing (decreasing α) or the DNA is becoming separated from the particle so that N is increasing. Indeed, these two descriptions are not mutually exclusive. One might imagine that the release of bound protein segments decreases the rigidity of the DNA while at the same time it frees a longer segment.

If the rigidity of the DNA of a core particle can change so readily with pH, then this response may have physiological significance. At the very least, workers in the field should carefully regard the effects of pH on any relevant chromatin activity under investigation.

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Alterations in Membrane Surfaces Induced by Attachment of Carbohydrates†

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ABSTRACT: We have examined the behavior of the dry phospholipid dipalmitoylphosphatidylcholine (DPPC) in the presence of several carbohydrate derivatives. These carbohydrate derivatives possess a hydrophobic portion which is incorporated directly into the DPPC membrane and a hydrophilic portion which places the carbohydrate structure at the membrane interface with the surrounding matrix. In the presence of these derivatives, the physical properties of the membrane are altered. These alterations are evident in changes observed in the phosphate and carbonyl vibrational modes of the phospholipid portion of the membrane. In addition, the phase transition behavior of the lipid is significantly altered as evidenced by a reduction in the gel to liquid-crystalline phase transition temperature. These results are consistent with those previously reported for free carbohydrates interacting with membranes in which a water replacement hypothesis has been used to explain the behavior. The attachment of carbohydrates to the membrane enhances these effects by localizing the agent responsible for these alterations at the membrane interface.

We have previously described the interaction of a class of synthetic glycolipids with membrane phospholipid vesicles composed of phosphatidylcholines and phosphatidylethanolamines (Goodrich et al., 1988; Goodrich & Baldeschwieler, 1987, 1988). These compounds have also been shown to exhibit a cryoprotective and lyoprotective action (Goodrich & Baldeschwieler, 1988). This latter property is manifested in an ability to prevent lipid mixing, increase in vesicular size, and leakage of vesicular contents following freeze-thawing and freeze-drying when present in mixtures with phospholipids in a 7:3 (lipid to derivative) mole ratio. In this paper, we examine specific alterations in lipid physical properties when dipalmitoylphosphatidylcholine (DPPC)[1] is dried in the presence

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[1] Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; FT-IR, Fourier transform infrared spectroscopy; IR, infrared spectroscopy; TRE, trehalose; TEC, triethoxycholesterol; TEC-Mal, maltosyltriethoxycholesterol; CHOL, cholesterol; L_α, liquid-crystalline lamellar phase; L_β, gel lamellar phase.

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