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The Structure of Chromatin: Interaction of Ethidium Bromide with Native and Denatured Chromatin†

J. Paoletti,‡ B. B. Magee, and P. T. Magee*

ABSTRACT: The binding of ethidium bromide, as monitored by fluorescence enhancement, to chromatin prepared by nuclease digestion has been compared with the binding of the dye to sheared chromatin. The nuclease preparation (native chromatin) is characterized by a high affinity region of the Scatchard plot (r = 0–0.025, $K_1 = 1 \times 10^6$ M^{-1}), a transition (r = 0.025–0.05), and a low affinity region (r = 0.05–0.12, $K_2 = 3 \times 10^5$ M^{-1}). The final amount of ethidium bromide bound per base is 0.12 as compared with 0.20 for free DNA. Sheared chromatin has the two regions of high and low affinity ($K_1 = 2 \times 10^6$ M^{-1}, $K_2 = 5 \times 10^5$ M^{-1}) as originally shown by Angerer and Moudrianakis (1972), but the transition is much reduced or absent. Binding of the dye to native chromatin is independent of salt at concentrations ranging from 0.2 mM EDTA to 10 mM Tris-Cl, 10 mM NaCl, 0.2 mM EDTA, while sheared chromatin and DNA both bind ethidium bromide electrostatically as well as by intercalation at the low salt concentration, leading to extensive energy transfer. Thus the phosphate groups in native chromatin are unavailable to external cations even at very low salt. Polarization of fluorescence of ethidium bromide intercalated into native chromatin at low r is very high, indicating a highly rigid structure. As r approaches 0.02, there is a very rapid depolarization; at r = 0.03, the polarization is no greater than that of the dye intercalated into DNA. Depolarization is not due to energy transfer. The Scatchard plot derived for the bulk preparation of native chromatin is very similar to the one derived for the monomer ν body. These results indicate that the DNA in native chromatin is in a very rigid form, with its phosphate anions neutralized by structural components, not by free salt. Ethidium bromide intercalation appears partially to disrupt this structure, perhaps by unwinding, leading to slight changes in its properties.

Chromatin in the nucleus appears to be a highly organized structure. The basic unit of this structure is a core of histones (H2A, H2B, H3, and H4) associated with 200 base pairs of DNA (Kornberg and Thomas, 1974; Noll, 1974; Van Holde et al., 1974; Burgoyne et al., 1974; Oudet et al., 1975; Senior et al., 1975).

In such a structure, the DNA is under constraints which may be similar to the topological constraints existing in circular DNA. It has been shown (Oudet et al., 1975; Griffith, 1975; Germond et al., 1975) that the association of the DNA with the histone core, during chromatin reconstitution, alters the supercoiling of a circular DNA. So it is conceivable that, in the

† From the Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut 06510. *Received June 14, 1976.* This work was supported by United States Public Health Service Grants GM 19481 and GM 21012.

‡ Present address: Laboratoire de Pharmacologie Moleculaire No. 147 du CNRS, Institut Gustave Roussy, 94800 Villejuif, France.

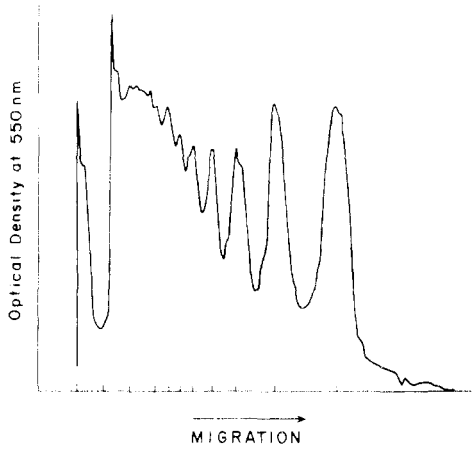

FIGURE 1: Polyacrylamide gel electrophoresis of DNA extracted from nuclease digested chromatin. DNA was extracted as described by Noll (1974). The material was subjected to electrophoresis on 2.5% polyacrylamide–0.5% agarose in tubes 0.6 × 8 cm. After staining and destaining (Experimental Section), the gel was scanned at 550 nm with a Gilford linear transport device.

chromatin, the association of the DNA with the histones causes a supercoiling of the DNA, and a model explaining the origin of the supercoiling of DNA in chromatin has been proposed (Crick and Klug, 1975). Such constraints in the structure of the DNA could play an important role in the biological properties of the chromatin, for example, at the level of the transcription or replication of the eukaryotic genome (Crick, 1971).

A number of studies involving the use of an intercalating drug to study the structure and the properties of chromatin have been reported (Lawrence and Louis, 1972; Angerer and Moudrianakis, 1972; Lurquin and Seligny, 1972; Williams et al., 1972; Angerer et al., 1974; Lapeyre and Bekhor, 1974). All these workers used chromatin whose extraction included mechanical shearing. It has been pointed out (Noll et al., 1975) that such procedures cause changes in the structure of the chromatin. These changes could be related to a displacement of the DNA along the histone core, due to the breakage of the noncovalent bonds responsible for the histone–DNA interactions. Therefore, it appeared to us that, if any supercoiled structure of the DNA exists in chromatin, due to a topological constraint, it would most likely have been disrupted by shearing and would not have been observed in previous studies.

One way to test for structural constraints in the DNA of the chromatin is to look at the intercalation of ethidium bromide in the DNA of chromatin, this intercalation being sensitive to the tertiary structure of the DNA (Le Pecq, 1972). In this report, we describe the effects of the method of purification of chromatin on the binding of ethidium bromide and discuss the binding in relation to current models of chromatin structure.

As reported earlier (Paoletti et al., 1976), we find two kinds of intercalating sites, one of high affinity and the other of low affinity, as previously described for sheared chromatin; but in chromatin prepared according to Noll et al. (1975), the passage from one type of binding to another is characterized by a sharp transition in the Scatchard plot of the binding. This transition, which reflects a cooperative effect in the binding, can be due either to an increase of affinity of the drug for the chromatin or to the appearance of new binding sites for intercalation. The effect, specific to "native" chromatin, is still present when we look at the binding of the drug to the monomeric unit of the chromatin. We interpret this transition as a loosening of the association between the DNA and the histone core, possibly caused by an unwinding of the DNA helix.

Experimental Section

Cell Line. WI-L2 is a nearly diploid human lymphoblastoid line from the Wistar Institute. It was grown as previously described (Magee et al., 1975).

Chromatin Purification. The cells, after centrifugation at low speed for 10 min, were washed twice in a saline solution and then suspended at a concentration of 10^7 cells per ml in buffer A (15 mM Tris[1] (pH 7.4), 60 mM KCl, 1.5 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 15 mM β-mercaptoethanol, 67 mM phenylmethylsulfonyl fluoride). Nonidet P-40 was added at a final concentration of 0.5%. After incubation for 1 min, the suspension was centrifuged and resuspended in 0.34 M sucrose–buffer A.

The suspension, containing mostly nuclei, was sedimented and the pellet suspended at a concentration of 10^8 nuclei per ml in 0.34 M sucrose–buffer A. The suspension was made 1 mM in $CaCl_2$ and digested with staphylococcal nuclease (1 μg/ml) for 10 min. The reaction was stopped by addition of EDTA to a final concentration of 2 mM, followed by chilling on ice. After centrifugation, the nuclear pellet was suspended with a Pasteur pipet in 0.2 mM EDTA and centrifuged at 4000 rpm for 10 min. The supernatant will be referred to as nuclease-extracted chromatin.

The size distribution of the DNA after extraction, as determined by gel electrophoresis (see details below), is shown in Figure 1.

Further purification was achieved by centrifuging the crude chromatin through a sucrose gradient (5–30%) for 18 h at 25 000 rpm in an SW27 rotor. These gradients show a pattern of bands corresponding to the monomeric, dimeric, etc. subunits of the chromatin. When needed, the material of each band was repurified in the same type of gradient.

Mechanically sheared chromatin was extracted as previously described (Magee et al., 1975).

Gel Electrophoresis. The distribution of the DNA length of the chromatin was determined by gel electrophoresis as described (Noll et al., 1975), followed by staining using Stains-all and destaining by diffusion against water (Sollner-Webb and Felsenfeld, 1975).

Binding Studies. Fluorescence studies of the binding of the ethidium bromide (EtBr) were carried out using a commercial apparatus (Hitachi Perkins-Elmer). The excitation wavelength was 520 nm, and emission was monitored at 600 nm.

We used two fluorescence cells (Precision Cells, Inc.): one with 3 ml of buffer and the other with 3 ml of chromatin solution (0.04 mM of DNA) in the same buffer. The EtBr was then added to each cell with a 5-μl pipet. The stock solution of EtBr was 0.023 mM. The final concentration of dye in the cuvettes was kept below 0.0015 mM in order to avoid any inner filter effect. When higher dye/polynucleotide ratios were desired, we lowered the starting concentration of chromatin. The buffers used were either: 10 mM Tris (pH 7.9), 10 mM NaCl, 0.2 mM EDTA, or 0.2 mM EDTA (pH 7). (The light scattering, if any, due to the chromatin was determined in the solution without EtBr and subtracted from the subsequent measurements. Usually at the chromatin concentration we used, no effect of light scattering was detected.) From measurements in these two cuvettes we obtain, for each concentration of dye, the fluorescence intensity of the free EtBr (I_f) and the fluorescence intensity of the bound dye (I_b).

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

Prior to the experiment we determined the fluorescence intensity of a solution of EtBr and the fluorescence of the same concentration of dye bound to a large excess of DNA (P/D = 5000). The ratio of these two fluorescence intensities (V) was used to determine the concentrations of bound and free EtBr for each point of the binding plot (Le Pecq and Paoletti, 1967).

If I_f is the fluorescence intensity of the free dye, VI_f is the fluorescence of the same concentration of EtBr completely bound to the DNA. The fraction of dye bound to the DNA in the experimental conditions is then $(I_b - I_f)/[(V-1)I_f]$ and the concentration of bound EtBr is equal to $C_0 = [(I_b - I_f)/(V - 1)I_f]C_t$ where C_t is the total concentration of ethidium bromide in the medium. The concentration of free ethidium is then: $C_f = C_t - C_b$.

From these data, we can plot the Scatchard equation (Scatchard, 1949) $r/c = K(n - r)$, where $c = C_f$ and $r = C_b/(\text{DNA})$; K is the affinity constant for the binding and n the number of dye molecules bound per nucleotide at saturation of dye.

Polarization of Fluorescence. These measurements were carried out in the laboratory of Dr. L. Stryer. The spectrofluorimeter we used was an apparatus built in the laboratory and has been previously described (Stryer, 1965).

The excitation monochromator was set at 520 nm and the emission read through a Corning 3-66 filter which transmits only wavelengths greater than 550 nm. Two polarizers (Polaroid Corp.) were mounted in the pathway of the light, one for the excitation, the other for the emission.

The components of the polarized emitted light, I_{vv}, I_{vh}, I_{hv}, and I_{hh}, were measured, and the coefficient of polarization was taken as equal to $p = (I_{vv} - I_{vh}t)/(I_{vv} + I_{vh}t)$, where $t = I_{hv}/I_{hh}$ is a constant of the system under the conditions of the measurements. Under our conditions, t was found equal to 1.02.

As in the case of the binding studies, the components of the fluorescence were measured for the free ethidium and for the dye bound to chromatin. The concentration of DNA was equal to 1.06 mM and the stock solution of EtBr had a concentration of 2.3 mM.

For each point, 5 μl of dye was added to 3 ml of buffer or chromatin solution, and the fluorescence intensities of the components of the emitted light were measured. At these concentrations of DNA, the correction due to the free ethidium could be neglected (Paoletti and Le Pecq, 1969).

A blank consisting of 3 ml of a solution of chromatin at a concentration of 1.06 mM was diluted with 5 μl of buffer at each measurement in order to correct for light scattering.

Fluorescence Lifetime. The fluorescence lifetimes were measured with an instrument built in the laboratory of Dr. Stryer and previously described (Yguerabide et al., 1970). For the excitation, we used a Corning 7.54 filter and, for the emission, a Corning 3.66 filter.

Reagents. The ethidium bromide was purchased from Calbiochem. It gives a unique fluorescent spot upon thin-layer chromatography (Le Pecq, 1972). The concentrations of ethidium bromide solutions were determined spectrophotometrically at 480 nm using an extinction coefficient of 5.45×10^{-3}. NP40 was a gift from Shell Chemical Co.

Micrococcal nuclease was purchased from Worthington.

Results

Binding Studies. The results of the binding of ethidium bromide to the sheared and DNase extracted chromatin are shown in Figure 2. The EtBr in these conditions (10 mM Tris-HCl (pH 7.9), 10 mM NaCl, 2 mM EDTA) binds the chromatin at two different sets of sites, one set with a high affinity (of the order of magnitude of the affinity for DNA), the other one with a lower affinity.

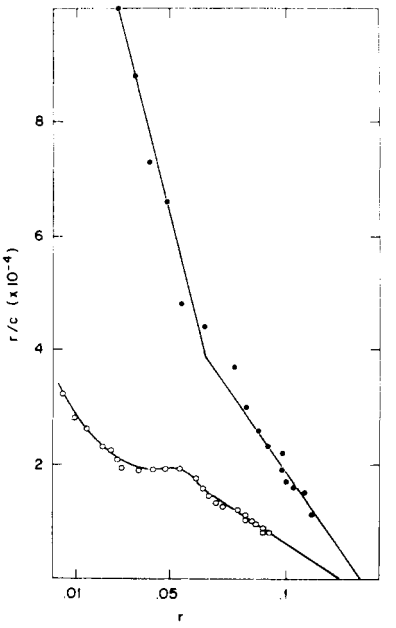

FIGURE 2: Scatchard plots of spectrofluorimetric titrations. (○) Chromatin prepared by in situ digestion of the nuclei; (●) chromatin prepared by mechanical shearing. The buffer was Tris (10 mM), pH 7.8, NaCl (10 mM), EDTA (1 mM).

These two kinds of sites have been reported previously (Angerer and Moudrianakis, 1972; Lurquin and Seligny, 1972; Angerer et al., 1974). The binding of the nuclease-extracted chromatin also shows two types of binding sites but, for this material, the passage from one type of site to the other is characterized by a striking transition. This transition occurs at a value of r of about 0.035 to 0.055 (EtBr/nucleotide). Such a transition could represent a cooperative effect on the binding of the ethidium to the second class of sites as a consequence of the saturation of the first class of sites. The value of r at saturation of EtBr is equal to 0.12 for the nuclease-extracted chromatin and 0.13 for the sheared chromatin. These values can be considered as equivalent, within the limits of the experimental error.

The respective affinities of the two types of sites are difficult to evaluate exactly due to their mutual interference (Klotz and Hunston, 1971; Angerer et al., 1974). However, by extrapolating to values of r where one or the other type of sites has little effect, we can estimate them to be: $K_1 = 1 \times 10^6\ M^{-1}$ and $K_2 = 3 \times 10^5\ M^{-1}$ for the nuclease treated chromatin and $K_1 = 2 \times 10^6\ M^{-1}$ and $K_2 = 5 \times 10^5\ M^{-1}$ for the sheared chromatin. The affinity of the EtBr for the DNA under the same conditions is $K = 3 \times 10^6\ M^{-1}$.

The nuclease-extracted chromatin was resuspended in 0.2 mM EDTA after extraction (see Experimental Section). For the binding experiment, the sample was dialyzed against the Tris-NaCl buffer. To see whether this step alters the structure of the chromatin (due to higher salt concentration or protease action), we performed a binding experiment immediately after extraction of the chromatin in 0.2 mM EDTA (pH 7). The results are shown in Figure 3.

For the DNase-extracted chromatin, there is no apparent effect of low salt. The Scatchard plot is quite comparable to that obtained with a Tris-NaCl buffer. The same transition

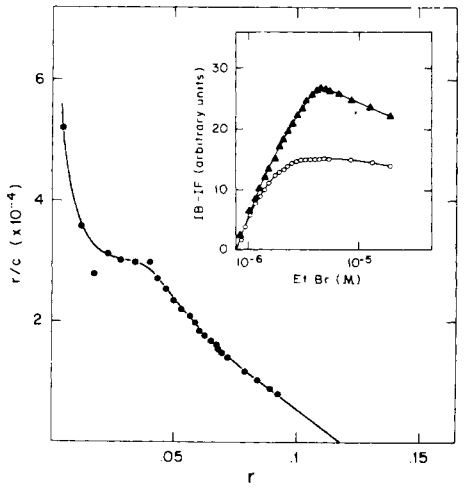

FIGURE 3: Scatchard plot of the [EtBr] binding in 0.2 mM EDTA (pH 7) to nuclease digested chromatin. (Inset) (▲) Fluorescence increment due to the binding of ethidium bromide to native DNA (0.2 mM EDTA (pH 7)). (○) Fluorescence increment due to the binding of ethidium bromide to mechanically sheared chromatin (0.2 mM EDTA (pH 7)). Note the dimunition of the fluorescence when the total concentration of ethidium bromide reaches 7×10^{-3} mM for DNA and 6×10^{-3} mM for chromatin.

between the high affinity and low affinity sites is apparent, and the same value of r at saturation of ethidium (0.12) is found. The affinity constant of the ethidium for the second class of sites was the same as before, but the constant for the first class of sites was higher: $K_1 = 3 \times 10^6\ M^{-1}$. However, under these conditions, the Scatchard plots of the binding of the dye to DNA and sheared chromatin are meaningless due to the strong influence of the external binding of ethidium bromide (Inset, Figure 3). This binding results from the electrostatic attraction of the positively charged dye by the phosphate groups of the polynucleotide (Le Pecq and Paoletti, 1967). The transfer of energy from the externally bound molecules of dye to the intercalated ones causes a decrease in the intensity of fluorescence.

We interpret these results to mean that the DNA, in the nuclease-extracted chromatin, is very tightly bound to the chromosomal proteins; neutralization of the phosphate groups prevents the electrostatic binding of the dye. The difference in affinity observed for the first class of sites indicates that these sites occur on less tightly bound DNA and are sensitive to the salt effect. By extrapolating the first part of the binding curve to a value of $r/c = 0$ and assuming we have a linear relation between r/c and r, we obtain a value of $r = 0.03$. This value is only approximate but allows an estimate of the fraction of DNA which has a high affinity for the ethidium bromide. This fraction represents around 25% of the total DNA in the chromatin. The model of chromatin now generally accepted is based upon a subunit of DNA of about 200 base pairs. Assuming that all the subunits are homogeneous for the binding of the dye, we can calculate the maximum amount of DNA with a high affinity to be of the order of 50 base pairs. This value agrees with the previous determinations of the amount of DNA differentially susceptible to nuclease attack (Van Holde et al., 1974). In comparison, the high affinity sites in the sheared chromatin represent a higher percent of the total DNA, about 50%. One explanation for this discrepancy is that the process of shearing, which is strong enough to break the phosphate–sugar backbone of the DNA, may also disrupt the noncovalent binding between the DNA and the chromosomal proteins, causing their sliding one upon another packing the ν bodies more closely together, and releasing more DNA for the ethidium bromide binding.

Another striking result is the apparent change of structure of the nuclease-extracted chromatin upon binding of the dye, as indicated by the cooperative nature of the binding process for values of r between 0.025 and 0.05. These values of r represent the intercalation of 10 to 20 molecules of EtBr per 200 base pairs, that is to say, per subunit.

Ethidium bromide is known to unwind the DNA, causing a releasing of the superhelical turns in circular supercoiled DNA (Bauer and Vinograd, 1968; Crawford and Waring, 1967; Bujard, 1968). One structure in chromatin which could be affected by the dye would be a supercoiled DNA. The unwinding effect of the intercalation might cause a disruption in the nucleosome–DNA interactions, leading to a general loosening of the structure and increasing the affinity of the DNA for EtBr. This would explain the cooperative nature of the second part of the Scatchard plot. If this interpretation is correct, then the supercoiling seems in some way to be intimately involved in the association histone–DNA. The fact that the DNA in the chromatin must be folded or supercoiled has been demonstrated by different workers (Rill and Van Holde, 1973; Oudet et al., 1975). Furthermore, a model relating the supercoiling of the DNA and its association with the histone core has been proposed (Crick and Klug, 1975).

Polarization of Fluorescence and Fluorescence Lifetime. If the DNA in the chromatin is topologically constrained, we should be able to detect it by looking at the polarization of fluorescence of the ethidium bromide intercalated in the chromatin. The polarization coefficient of a fluorescent chromophore reflects the rotation this chromophore undergoes between excitation and emission. For ethidium bromide, it has been shown that the coefficient of polarization in a rigid medium (infinite viscosity) is equal to 0.415 (Wahl et al., 1970; Paoletti, 1971). If the molecule undergoes any rotational motion during the excitation and the emission, we will observe a depolarization of the emitted light, causing a decrease in the value of the polarization coefficient. When EtBr is intercalated in the DNA, it is subject to the rotation of the base pairs around the long axis of the helix, and its coefficient of polarization decreases from 0.415 to 0.320 (Wahl et al., 1970). Depolarization can be attributed to rotational motion as long as there is no interference by a transfer of energy between dye molecules. If such a transfer occurs, the depolarization will reflect not the motion of the dye but the rotation of the emitted light due to the angle between two adjacent molecules of dye. In order to eliminate this effect, we have to determine the polarization by extrapolation to $r = 0$. When the dye is intercalated in a compact DNA, for example in the bacteriophage λ, the polarization of fluorescence is increased to a value of 0.410 (Paoletti, 1971). In sheared chromatin, it has been shown that the polarization of fluorescence of the intercalated EtBr is higher than in DNA (Angerer et al., 1974). We have measured the polarization coefficient of the ethidium intercalated in nuclease-treated chromatin at different dye/polynucleotide ratios and at different viscosities. The results are shown in Figure 4. For low values of the ratio of bound dye/nucleotide, the polarization of fluorescence of the chromatin-bound EtBr is higher than that observed for the DNA-bound dye. We determine, under these conditions, a value of $p = 0.415$ for the chromatin and 0.320 for the DNA. The value for the DNA is in agreement with that previously found (Paoletti and Le Pecq, 1971).

As r increases from 0 to 0.02, the polarization of the emitted light decreases sharply and then a gradual depolarization

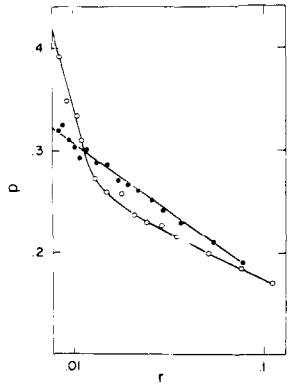

FIGURE 4: Variation of the coefficient of polarization (p) as a function of r for DNA (●) and nuclease-extracted chromatin (○).

comparable to that found for the DNA occurs. Sheared chromatin is very similar to nuclease-treated chromatin, except that the slope is smaller at low r and that p extrapolates to 0.370 for $r = 0$ (data not shown). The range of rapid depolarization (from $r = 0$ to $r = 0.02$) coincides with the range of high affinity in the binding isotherms. This result implies that the DNA in the chromatin is unable to undergo any internal rotational motion. The striking depolarization for low values of r, much greater for chromatin than for DNA, could be explained by either of two interpretations.

(a) The portion of DNA with a high affinity for the dye is quickly saturated. A strong depolarization of the fluorescence then occurs due to an energy transfer from one molecule of dye to another, either a transfer from intercalated dyes to externally bound ones or transfer between intercalated dyes, although the results with low salt seem to exclude external EtBr binding (Figure 3).

(b) In the process of binding the ethidium bromide, the DNA is partially or fully released from the histones and the base pairs recover their capacity to undergo rotational motion.

The second hypothesis can be tested by looking at the polarization of fluorescence of the bound ethidium bromide at high viscosity. If the depolarization is due to an energy transfer, we should observe no difference in the values of the polarization at low and high viscosity. However, if the depolarization is due to a motion of the base pairs of the DNA, we should, by increasing the viscosity, prevent the abrupt depolarization accompanying the binding and find only a depolarization parallel to that in a DNA–EtBr binding experiment. The results of such an experiment are shown in Table I. The coefficient of polarization is not changed when the viscosity of the medium is increased, a result which excludes any important change of the rotational properties of the base pairs. The depolarization observed during the saturation of the first type of sites must thus be due to a transfer of energy between molecules of ethidium bromide, indicating a clustering of the dye in small regions of the DNA.

We can now ask whether the energy transfer associated with the high affinity binding is due to a transfer between intercalated ethidium bromide, or to a transfer from intercalated to externally bound EtBr. If the transfer occurs from sites of intercalation to external sites, we should observe a diminution of the fluorescence lifetime of the intercalated ethidium bromide as the ratio of dye to polynucleotide increases. This diminution is due to the fact that the transfer of energy to nonfluorescent (nonintercalated) dye molecules provides a second path for the decay of fluorescence.

TABLE I: Variation of the Degree of Polarization of Dye Bound to Chromatin As a Function of r and Viscosity.

r	% Sucrose (w/v)	P
5×10^{-3}	0	0.35
5×10^{-3}	30	0.35
5×10^{-3}	60	0.35
2×10^{-2}	0	0.27
2×10^{-2}	30	0.28
2×10^{-2}	60	0.28
5×10^{-2}	0	0.23
5×10^{-2}	30	0.23
5×10^{-2}	60	0.23
1×10^{-1}	0	0.18
1×10^{-1}	30	0.20
1×10^{-1}	60	0.20

TABLE II: Variation with r of the Fluorescence Lifetime of Ethidium Bromide Bound to DNA or to Chromatin.

	r	Lifetime (ns)
DNA	9.3×10^{-1}	21.7
Chromatin	3×10^{-3}	21.4
	4×10^{-2}	19.8
	1.2×10^{-1}	21.4

We have looked at the lifetime of the ethidium bromide bound to the chromatin as a function of increasing values of r. The results are shown in Table II. The lifetime of fluorescence of the bound ethidium is very similar whether r is low (0.03) or high (0.12). This indicates that, even at saturating concentrations of ethidium bromide, the DNA in the chromatin has a very small number of external sites available to the dye. This may be due to neutralization of the phosphate groups by the basic residues of the histones. However, for a value of $r = 0.04$, corresponding to the saturation of the sites with a high affinity for the dye, there is a slight decrease (21.4 to 19.8 ns) in the value of the lifetime, suggesting that there is some energy transfer when the high affinity sites on the chromatin are saturated, between intercalated dye and molecules bound to the phosphate groups of the DNA.

The results of the polarization of fluorescence and lifetime measurement determinations confirm the presence, in nuclease-prepared chromatin, of two different types of sites. The strong depolarization characterizing the intercalation of the EtBr for values of r smaller than 0.025 indicates that the DNA with a high affinity for the dye ("open DNA") is present in the chromatin as stretches separating regions where the DNA is protected by the histones (and thus has low affinity for EtBr) since the bound molecules of the dye must be within 17 Å (five base pairs) to transfer energy efficiently (Paoletti and Le Pecq, 1971). The total length of the "open" DNA is around 50 base pairs. We have assumed in calculating this value that the structure of the chromatin is a repetitive one, and that the binding of the ethidium bromide to the subunit is the same as the binding to the intact structure. In order to verify this assumption, we have purified the monomer of chromatin and looked at the binding of the ethidium bromide. The monomeric subunit of the chromatin was purified through a sucrose gradient as previously described (Noll, 1974). We collected the fractions corresponding to the monomer and, after dialyzing against a solution of 0.2 mM EDTA, we looked at the binding

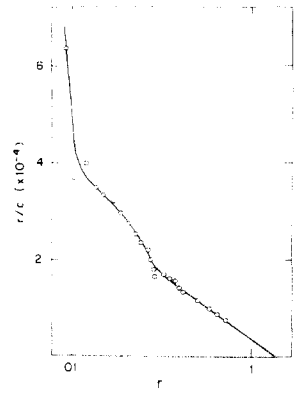

FIGURE 5: Scatchard plot of the binding of ethidium bromide to monomers of chromatin. Same conditions as Figure 3.

of the ethidium bromide (Figure 5). The monomer of chromatin binds the ethidium bromide in a way very similar to total chromatin except for a higher affinity in the initial part of the plot. The same cooperativity appears for a value of r between 0.025 and 0.05, and the value of r at saturation of ethidium is the same (0.12) as for the total chromatin. This result indicates that the constraints observed in the chromatin are found at the level of the monomer and can probably be related to the mode of wrapping or folding of 200 base pairs of DNA along the histone core.

Discussion

Chromatin prepared by digestion in situ of nuclei with micrococcal nuclease presents some interesting structural properties. It is now generally admitted that the chromatin is built as a repeating subunit formed by the association of 200 base pairs of DNA with a histone core (Kornberg, 1974; Kornberg et al., 1975; Van Holde et al., 1974; Oudet et al., 1975).

The DNA in such a structure should be in a compact form and subject to constraints similar to those in supertwisted circular DNA. Such a compact structure has already been demonstrated in the case of the PS (or low salt soluble) fractions of the chromatin (Rill and Van Holde, 1973). The results we report here support this idea.

We originally reported that the Scatchard plots for the binding of ethidium bromide to chromatin are biphasic (Paoletti et al., 1976). The passage from one type of binding to the other is marked by a cooperative effect; the total amount of ethidium bromide bound to the chromatin at dye saturation represents 1 molecule/8 nucleotides, half the value found for the DNA in solution. The binding of the dye to the high affinity sites is accompanied by a strong depolarization of the fluorescence and a slight decrease in the fluroescence lifetime. The coefficient of polarization of the ethidium bound to the chromatin at low values of r is higher than the coefficient we measure for the ethidium bound to DNA. By extrapolating the values of p to $r = 0$, we obtain a value of 0.415. The binding of the ethidium to a monomeric subunit of chromatin is comparable to the binding to the multimeric structure.

The DNA in chromatin, therefore, has two different kinds of binding sites for ethidium bromide. One part of the DNA, representing around 50 base pairs, binds the dye with a high affinity, comparable to the affinity of naked DNA. The dye saturates the sites in this DNA, causing a significant depolarization of the fluorescence due mainly to an energy transfer between intercalated molecules. The phosphate groups of this DNA are also able to bind some EtBr by electrostatic interaction, as shown by the slight decrease in the value of the fluorescence lifetime, when the first sites are saturated.

The ethidium bromide binds to the remaining DNA (around 150 base pairs) with a lower affinity. We attribute this lower affinity (comparable to the affinity of naked DNA in high salt) to neutralization of the phosphate groups by histones. Such an interpretation is supported by the fact that this binding is unaffected by an increase of the salt concentration. The value of r at saturation is half the value for naked DNA. Two mechanisms could explain this result: either half of the DNA is covered with proteins and protected from the ethidium bromide, or the DNA is strongly bound to the histone core at discrete points and has to be wound to accommodate the local unwinding due to the intercalation of the ethidium. Under the latter hypothesis, the structure of this DNA would be analogous to the structure of circular DNA, which cannot accommodate more than 1 EB molecule/8 nucleotides at saturation of dye. The binding of the EtBr to the high affinity sites induces a cooperativity in the binding to the second types of sites. This cooperativity is characterized by an apparent increase of the affinity of the dye for the chromatin when the high affinity sites are saturated. We suggest the following mechanism for this increase in the affinity. At very low concentrations of ethidium bromide, small regions of DNA (the high affinity part of the chromatin) are able to intercalate the dye. This intercalation unwinds the DNA, relaxing its structure and allowing the remaining DNA, which now is less tightly bound to the histones, to accommodate more ethidium. The binding of more ethidium causes the structure to be wound up to the point, as in circular DNA, where the constraint is such that the DNA cannot accommodate any more ethidium. Such a process could explain the shape of the binding isotherms.

Assuming that unwinding of the DNA is responsible for its partial release from the histone core, we can calculate the number of turns involved in this process. The unmasking of the DNA is completed at an r value of 0.04, which represents the binding of 1 molecule of EB per 12 base pairs, or 15 molecules per 200 base pairs. Intercalation of one molecule of EB unwinds the DNA by 26° (Wang, 1974). We can then estimate the unwinding necessary to release the DNA from the histone to be 390°. This figure is in good agreement with the results of Germond et al. (1975), who showed that each histone core particle was associated with one supertwist.

In the "kinky helix" model Crick and Klug (1975) propose the existence of kinks every ten base pairs along the double helix of DNA associated with the histone core. These kinks would be responsible for inducing a supertwisted structure in the DNA, with the number of superturns per subunit equal to $19\theta/360$, where θ represents the unwinding angle due to one kink. Our results, taken together with those of Germond et al. (1975), indicate that the model of Crick and Klug is a plausible one.

The last interesting feature of our results is that the constraints we observe cause a complete immobilization of the DNA so that it is unable to undergo any internal motion.

The binding of the ethidium to sheared chromatin gives the same type of results with the exception of a higher fraction of DNA with high affinity for the dye and a smaller polarization of fluorescence for $r = 0$. However, we can see in Figure 2 a slight cooperativity in the binding of the EB. These results are in agreement with the hypothesis that shearing causes the DNA to slide along the histone core. This sliding may uncover parts of the DNA previously protected by the histones, partially releasing the polynucleotide chain from the histones and allowing this DNA to be more mobile. The binding of the dye

to this DNA would be expected to have the characteristics (high affinity, low polarization) that we see in sheared chromatin. Nevertheless, the shearing of the chromatin does not completely destroy its structure since it is possible to isolate nuclease-resistant fragments from chromatin solubilized by mechanical shearing (Van Holde et al., 1974), and since the binding of the EtBr to that type of structure can give cooperativity similar to that we observed in nuclease-extracted chromatin.

The supertwisting of the DNA in chromatin and the possibility of modulating the strength of the association between the DNA and the histone core with an unwinding agent may play an important role in the biological properties of the chromatin. We can suppose that unwinding proteins are associated with the chromatin. These proteins might have the same effect as the ethidium bromide, that is, an eventual loosening of the DNA from the histones, and so could play an important role in the regulation of the replication or the transcription of the eukaryotic genome.

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