Ethidium Bromide as a Probe of Conformational Heterogeneity of DNA in Chromatin. The Role of Histone H_1†

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ABSTRACT: The accessibility and the tertiary structure of the DNA inside chromatin were studied by using ethidium bromide (EB) as a fluorescent probe. The exclusion model of binding was refined by introducing a parameter α ($0 < \alpha < 1$) which measures the accessibility of the DNA and by taking into account when necessary the existence of two sets of binding sites. We were thus able to fit predicted and experimental isotherms and then to describe completely EB binding to native or partially histone depleted chromatin under various conditions. In native chromatin 95% of the DNA ($\alpha = 0.95$) appears to be accessible to EB but two sets of sites are present. The first one corresponds to $\alpha = 0.13$ and is characterized by an affinity constant which is higher by two orders of magnitude than that relative to pure DNA. The second set corresponds to $\alpha = 0.82$ and the corresponding binding constant is only three or four times lower than that of pure DNA. The sites with high affinity are still present after treatment with formaldehyde but disappear after removal of histone H_1. By comparison with chromatin treated with deoxycholate or with artificial complexes between H_1 and DNA, high affinity sites were found only when all of the histones are bound to DNA. An α value around 0.8 is still obtained in 1 M NaCl treated chromatin, pointing to the fact that histones H_3 and H_4 are preventing 20% of the DNA to intercalate EB.

Recent studies on chromatin have provided new information about the organization of chromatin components. Although the idea of chromatin subunits is rather an old one and comes from functional studies (Georgiev, 1973), the idea of structural subunits in chromatin have been extensively developed since the work on the nuclease digestion of chromatin either by nuclear Ca–Mg dependent nuclease of Hewish and Burgoyne (1973) or by micrococcal nuclease (Rill and Van Holde, 1973; Noll, 1974; Weintraub, 1975; Clark and Felsenfeld, 1974).

The idea of a subunit structure for chromatin largely simplifies the study on the arrangement of chromosomal components in the chromatin fiber since one can assume that, if a structural event arises in a subunit, it could arise in all the others. This seems to be the case for the association of the four structural histones (H_{2A}, H_{2B}, H_3, H_4) which are bound to the DNA in the form of a socalled "octamer" formed from two of each histones, the two histones H_3 and H_4 forming themselves a tetramer (Kornberg and Thomas, 1974; Thomas and Kornberg, 1975). Other forms of histone association have been found to occur in native chromatin, such as H_{2B}–H_4 dimers (Martinson and McCarthy, 1975), or H_{2B}–H_{2A} dimers (Van Lente et al., 1975). They demonstrate the propinquity of the four structural histones in the chromatin structure except for histones H_1.

This histone is considered to have no influence on the tertiary structure of the DNA in the subunit and most of the structural studies on the chromatin are performed on H_1-depleted chromatin. Nevertheless several studies have shown that histones H_1 or H_5 have an important role in biological function of chromatin such as transcription (Felsenfeld et al., 1974) or condensation of the chromatin fiber during the cellular cycle where important chemical modifications occur (Balhorn et al., 1972; Bradbury et al., 1974).

In a previous paper (Lawrence and Louis, 1974), we have pointed out a special influence of histone H_1 on the physicochemical state of the DNA, as followed by ethidium bromide (EB)[1] binding studies. In order to improve the study of the role of individual chromosomal components on the physicochemical state of the DNA, we have studied in more detail the binding of EB into the DNA in presence or absence of such components. Reevaluation of the binding equations, taking into account a more detailed description of the system, yields rather different conclusions from those previously published from other studies (Angerer and Moudrianakis, 1972; Lurquin and Seligy, 1972) or obtained with another probe (Lawrence and Louis, 1972). The presence in chromatin of two types of sites, for the intercalation of EB between base pairs, one of which has a much greater binding constant than that of pure DNA, strongly suggests structural heterogeneity of chromosomal DNA.

Materials and Methods

Chromatin is extracted from calf thymus by a modification (Lawrence, 1974) of the Zubay and Doty method (1959). Every blending step is minimized to avoid mechanical shearing and the integrity of chromatin fiber is controlled by means of electron microscopy according to the Bram and Ris method (1971).

Histone extraction is performed with sodium chloride by the method described by Ohlenbusch et al. (1967) and histones remaining on the pellet are analyzed by polyacrylamide gel electrophoresis by the method of Panyim and Chalkley (1969). The results are identical with those already described (Ohlenbusch et al., 1967; Lawrence and Louis, 1972) (see Table I).

The majority of nonhistone proteins are extracted from native chromatin by three washings with 0.35 M NaCl as described by Goodwin and Johns (1972).

Proteins are covalently linked to DNA by a formaldehyde fixation (Brutlag et al., 1969). A chromatin gel ($A_{260} \simeq 6$) is

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[1] Abbreviations used: EB, ethidium bromide; DNP, deoxynucleoprotein.

allowed to dialyze during 15 h against a triethanolamine buffer (0.01 M, pH 7.8) containing 1% formaldehyde. The triethanolamine and formaldehyde are then eliminated from the gel by dialysis against 10^{-3} M sodium chloride solutions. A reference solution of chromatin is submitted to the same treatment omitting formaldehyde and gives the same results as the standard.

Sodium deoxycholate extraction of histones is carried out by a modification of the Smart and Bonner method (1971). The original method gives rise to a dissociation–reassociation process during the extraction produced by the use of a sodium deoxycholate concentration approximately ten times higher than that required in the final solution.

The modified method consists of dispersing the chromatin pellet obtained at the end of the preparation procedure in the desired final concentration of a deoxycholate solution (i.e., 0.01, 0.03, 0.05, and 0.08 M depending on the histone to be extracted). This solution is allowed to equilibrate for 1 h, and is then centrifuged over a 2 M saccharose cushion in a 60 Ti Beckman rotor at 40 000 rpm during 15 h. The pellet is then solubilized in a 10^{-3} M NaCl solution buffered with 10^{-1} M Tris-HCl (pH 8), allowed to dialyze against this same solution to eliminate the deoxycholate, and then dialyzed against NaCl alone to eliminate the Tris buffer (see Table I).

F_1–DNA reconstituted complexes are made according to the step-dialysis method described by Touvet-Poliakow et al. (1970). Three protein/DNA ratios have been used, i.e., 0.1, 0.45, and 0.7.

Reassociation of Dissociated Proteins. A solution of chromatin ($A_{260} \simeq 6$) is allowed to equilibrate (12 h) by dialysis against the appropriate concentration of sodium chloride solution. When the equilibrium is reached, the reassociation is achieved by dialyzing the mixture against three changes of 10^{-3} M NaCl.

All protein/DNA ratios are determined by the Lowry method for estimation of proteins. DNA is estimated by its absorbance at 260 nm, and histones remaining on the pellet after saline extraction are analyzed by polyacrylamide gel electrophoresis (Panyim and Chalkley, 1969) after HCl extraction. RNA was always absent from our preparation.

Ethidium Bromide Binding. EB binding is measured essentially as described by Le Pecq and Paoletti (1967) with a Zeiss spectrofluorometer with two monochromators ($\lambda_{excitation}$ = 490 nm; $\lambda_{emission}$ = 590 nm). All measurements are made at room temperature (T 23 °C) with a solution containing 10^{-3} M NaCl. It is well known that the intercalation of EB into DNA (between two base pairs) enhances the fluorescent quantum yield but external binding does not. So the method used here, which is a measure of an increase of fluorescence, can give an estimation of the intercalated sites only. The complete treatment of the fluorescent data is described elsewhere (Lawrence, 1974) and all calculations are made with a desk computer.

Description of Binding Models

The binding of EB to DNA is well described by an equilibrium of this kind:

$$\text{DNA} + \text{EB} \rightleftharpoons \text{DNA–EB}$$

The problem of the choice of a model consists in determining the number of available binding sites on the DNA, and then to calculate the binding constant K.

As originally described by Le Pecq and Paoletti (1967) or by Waring (1965), the model was to suppose that a proportion of N sites on the DNA were available for binding with an uniform binding constant K. This gives rise to the well-known equation:

$$r/C = K(N - r) \tag{1}$$

which, in the Scatchard's plot, is represented by a straight line.

In this equation as in all the following, r is the amount of bound dye per *base pair* and C the concentration of free dye when equilibrium is reached. Scatchard plots of experimental data according to this equation result in an estimate of approximately 0.5 for the number of available binding sites on the DNA, i.e., that half of the DNA base pairs contributes to the binding. Such a result may be interpreted to represent binding occurring in two extreme ways: either one-half of the DNA molecule contributes to the binding while the other remains unbound, or ethidium bromide binds every other site. Any intermediate between these two states is possible. The model used here is unable to discriminate between them.

There are, however, no physical reasons for the binding to occur on DNA following the first extreme way described above and, to describe more faithfully a model in which the whole DNA molecule is implicated in the binding process, Crothers (1968) has proposed the excluded site model. The model is based on the assumption that the binding of an EB molecule (or more generally one ligand molecule) on a base pair excludes the binding on the n' first neighboring base pairs. This presumes different statistics for the calculation of the binding isotherm equation and the matrix method developed for the helix–coil transition description (Poland and Scheraga, 1970) may be applied to this model (Crothers, 1968). The resulting explicited equation is:

$$\frac{r}{C} = K\frac{(1 - n'r)^{n'}}{(1 - (n' - 1)r)^{n'-1}} \tag{2}$$

One can calculate according to this equation that the best fit of experimental data for DNA occurs for $n' = 2$, a result which is consistent with that obtained from the first model described, but which is now based on a real physical effect. The shape of a binding isotherm is now significantly different from that of the previous model and parameters obtained using this model are closer to those obtained by kinetic methods.

Finally, there is a case in which one may assume that there exists physical or chemical constraint on the DNA that prevents EB binding to certain sites. This is taken into account by supposing that a fraction α ($0 \leq \alpha \leq 1$) of the DNA molecule contributes to the binding, and consequently the remaining fraction $(1 - \alpha)$ does not. For example, this may be the case in nucleoproteic complexes where one can postulate that the binding of protein to DNA prevents the binding of EB to the same place. This has been proved on synthetic complexes between histone H_3 and DNA (Lawrence, 1974), where the apparent number of binding sites is considerably lowered. Introducing these new parameters into the isotherm calculation yields the following equation:

$$\frac{r}{C} = K\frac{(\alpha - n'r)^{n'}}{(\alpha - (n' - 1)r)^{n'-1}} \tag{3}$$

Best fit calculation (see below) with this equation shows that in nucleohistone complexes $n' = 2$ and α decreases as the amount of protein increases. $n' = 2$ means that binding on available DNA still occurs according to the excluded site model but the decrease of α means that a portion of the DNA is no longer available for EB binding. Once n' is determined, the values of α and K which best fit the experimental data can be calculated. The fraction of DNA chemically blocked for EB binding is $(1 - \alpha)$.

TABLE I: Chemical Composition of Various Samples Used and Prepared as Described in Material and Methods.[a]

Samples	PROT/DNA (w/w)	Approx. % of Original Histone Fraction				
		H_1	H_{2A}	H_{2B}	H_3	H_4
Chrom. (0.6 M) NaCl)	1.20	0-5	95-100	95-100	95-100	95-100
Chrom. (1 M NaCl)	0.85	0	5-10	5-10	80-90	80-90
Chrom. (2 M NaCl)	0.24	0	0	0	0	0
Chrom. (0.01 M DOC)	1.55	100	100	100	100	100
Chrom. (0.03 M DOC)	0.52	90-100	15-20	15-20	15-20	15-20
Chrom. (0.05 M DOC)	0.40	90-100	15-20	15-20	15-20	0
Chrom. (0.08 M DOC)	0.22	80-90		0	0	0

[a] For abbreviations used in this table, see footnote *a*, Table II.

TABLE II: Binding Parameters of the Samples Described in the Text.[a]

Samples	PROT/DNA (w/w)	K_1 (l. M^{-1})	α_1	K_2 (l. M^{-1})	α_2	K (l. M^{-1})	α
Chromatin	1.60	1.4×10^9	0.13	2×10^5	0.82		
Chromatin HCHO 1%		2.2×10^8	0.14	1.3×10^5	0.65		
Chromatin 0.6 M DR		2.3×10^9	0.17	1.5×10^5	0.80		
Chromatin 0.35 M	1.40	2.1×10^9	0.16	2.1×10^5	0.82		
0.6 M NaCl extracted	1.20					8.8×10^5	0.75
1 M NaCl extracted	0.85					9.0×10^5	0.81
2 M NaCl extracted	0.24					1.4×10^6	0.94
DNA ($\mu = 10^{-3}$)	0					2.14×10^6	1.0
DNA ($\mu = 10^{-1}$)	0					2.6×10^5	0.98
0.01 M DOC extracted	1.55	1.7×10^9	0.14	2×10^5	0.82		
0.03 M DOC extracted	0.52	8.5×10^6	0.39	4×10^5	0.64		
0.05 M DOC extracted	0.40					3.4×10^6	0.82
0.08 M DOC extracted	0.22					2×10^6	0.90
H_1-DNA	0.10					1.2×10^6	1.0
H_1-DNA	0.45					8.2×10^5	1.0
H_1-DNA	0.70					7.6×10^5	0.99

[a] Abbreviation used: chromatin HCHO 1%, chromatin treated with formaldehyde as described in the text; chromatin in 0.6 M DR, dissociated and reassociated chromatin in 0.6 M NaCl; chromatin 0.35 M, nonhistone-protein-depleted chromatin; NaCl extracted, chromatin extracted with various concentrations of NaCl; DOC extracted, chromatin extracted with various concentrations of sodium deoxycholate.

The situation is a little more complicated when binding of EB occurs onto two types of independent binding sites defined respectively by their intrinsic parameters: α_1, K_1 and α_2, K_2. Here it is more useful to express the binding ratios in the following way, derived from eq 3, where $n' = 2$:

$$r_1 = \frac{\alpha_1}{2}\left[1 - \left(1/\sqrt{1 + 4K_1C}\right)\right] \quad (4)$$

$$r_2 = \frac{\alpha_2}{2}\left[1 - \left(1/\sqrt{1 + 4K_2C}\right)\right] \quad (5)$$

The amount of measured bound dye is $r = r_1 + r_2$ and that of free dye is C.

The set of values of α_1, k_1 and α_2, k_2 which best fits the experimental data is determined by iterative calculation attributing values for α_2, K_2 to start the procedure.

Results

Figure 1 shows binding isotherms of EB to DNA with an ionic strength 10^{-3} M NaCl. The solid line represents the best fit obtained by the method described above. The value of α giving the best fit is of course 1, but the value found for K (Table II) is about half that found by Le Pecq and Paoletti (1967) as predicted by Bauer and Vinograd (1968). A higher ionic strength (10^{-1} M NaCl) does not change the value of α but lowers the binding constant as already described (Le Pecq and Paoletti, 1967; Waring, 1965).

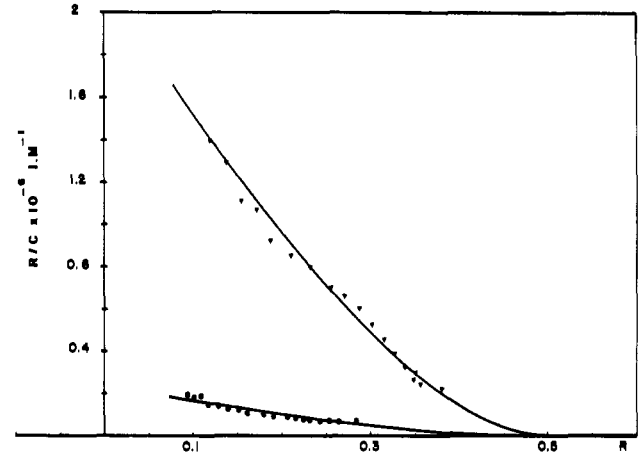

FIGURE 1: Binding isotherms of EB to DNA for two ionic strengths. (▼) 10^{-3} M NaCl; (●) 10^{-1} M NaCl. Solid lines represent the best fit curves as calculated from eq 2. R is the amount of bound dye per base pair; C is the concentration of free dye at equilibrium.

The binding isotherm of EB to chromatin (Figure 2) is very different from that of binding to DNA. As already described in a preliminary report (Lawrence and Louis, 1974), the binding takes place onto two types of independent binding sites. The treatment of the data was made according to the excluded

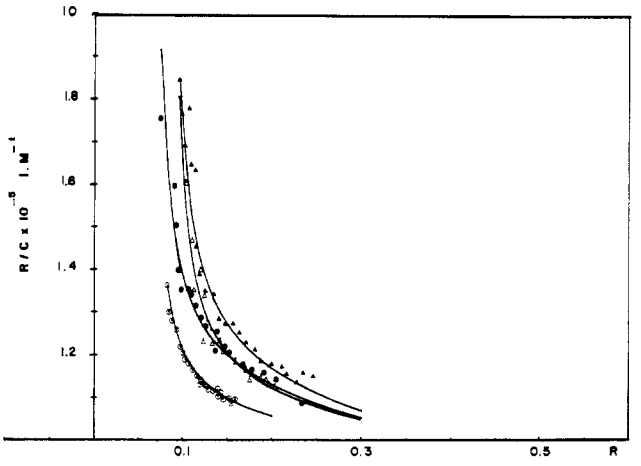

FIGURE 2: Binding isotherms of EB to chromatin (●); nonhistone-protein-depleted chromatin (▲); dissociated and reassociated chromatin in 0.6 M NaCl (△); and HCHO 1% treated chromatin (⊙). Solid lines represent best fit calculation from experimental data according a two-binding-site model as described in the text.

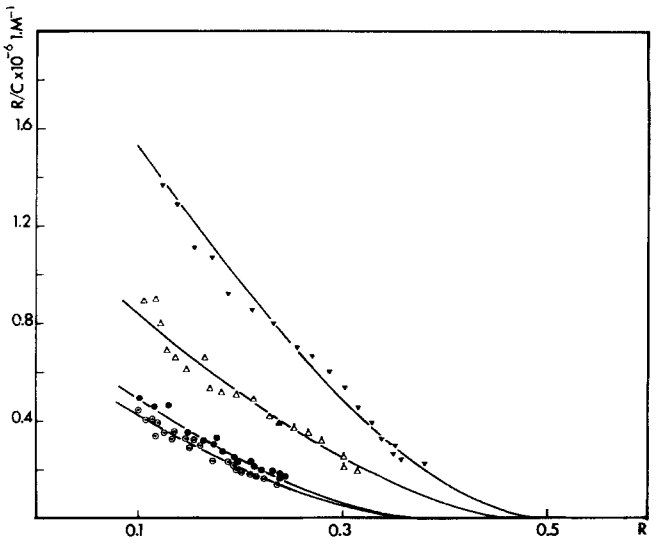

FIGURE 3: Effect of NaCl extraction of histones from chromatin on the EB binding isotherms. Chromatin extracted with: 0.6 M NaCl (⊙); 1 M NaCl (●); 2 M NaCl (△); and pure DNA (▼).

site model taking into account a restriction of the accessibility of EB to DNA.

One can see from the values tabulated in Table II that chromatin displays two kinds of binding sites. The first one involves only 13% of DNA and is characterized by a very high value of the binding constant (700 times higher than that of pure DNA). The second kind of binding sites involved 82% of the DNA and has a binding constant consistent with that expected from the ionic strength effect of histones and other proteins to the DNA (see Discussion).

It is interesting to estimate the role of the various chromosomal components in producing the special binding mode observed in chromatin. Figure 2 shows the binding isotherm obtained with nonhistone-protein depleted chromatin prepared as described in Material and Methods. The two-binding-site process is still present and the parameters are only slightly modified. These proteins seem not to be responsible for the presence of high affinity binding sites.

The situation is very different for the histone H_1. As shown in Figure 3, the extraction of this histone from chromatin by NaCl (0.6 M) induces the disappearance of the high affinity binding sites.

The remaining sites involve 75% of the DNA (i.e., 25% of the DNA is now blocked for EB binding) and the binding constant is higher than that of low affinity binding sites of chromatin (Table II). This situation is nevertheless reversible. If histone H_1 is reannealed to partial chromatin from the dissociating medium, one obtains a binding isotherm which is nearly superimposed on that of native chromatin (Figure 2). The number of high affinity binding sites is a little higher. This may be caused by an irreversible process of the dissociation of nonhistone proteins during the dissociation–reassociation process of histone H_1. We have shown in fact that the extraction of nonhistone proteins induces a slight increase in the number of high affinity binding sites. The α value (0.82), which corresponds to the second type of binding sites in the native chromatin, is slightly higher than that measured in H_1-depleted chromatin (0.75). If both the two types of binding sites are taken into account, the DNA appears to be more accessible inside the native chromatin than inside the H_1 depleted one. It was interesting to see if such a paradoxical situation still occurs after treatment with HCHO, in order to "prevent" any "sliding" or removing of histone molecules. In the formaldehyde reacted chromatin (Figure 2 and Table II), the two-binding-site process is still present. The number of high affinity binding sites is not significantly changed but their binding constant is reduced to 2.2×10^8. It is interesting to notice that only the number of low affinity binding sites is lowered (from 82 to 65%), indicating the proximity of protein residues to these sites as it was expected from the lower value of the binding constant, when compared with that measured with pure DNA.

Figure 3 and Table II show the effect of further histone removal from chromatin. The binding constant displays a monotonous variation except for the point representing the low affinity binding sites of chromatin. Since histone H_1 seems to play a very special role in the binding properties of chromatin, it was interesting to study EB binding to artificial complexes between this histone and pure DNA.

The result is shown in Figure 4 for three different protein/DNA ratios (0.1, 0.4, 0.7). At protein/DNA ratios as low as 0.1, the binding constant is already lowered by a factor of 2. But the two-binding-site process was never found even in the presence of the highest amount of protein.

The negative result suggests a possible role for the tertiary structure of DNA in chromatin and it was necessary to estimate the influences of histone H_1 in the presence or absence of such a structure. To achieve this, histones were extracted with sodium deoxycholate as described in Materials and Methods. Histones remaining on the partial nucleoproteic pellet after extraction were analyzed by gel electrophoresis. One can see from Table I the effect of increasing concentration of sodium deoxycholate. After the use of 0.03 M sodium deoxycholate, each of the five histones is still present but with a predominance of histone H_1. In 0.05 M deoxycholate, histones H_4 and H_3 have been entirely extracted and H_{2B} and H_{2A} partially extracted. In 0.08 M only H_1 remains bound to the pellet and this histone can be extracted in 0.1 M sodium deoxycholate.

The behavior of EB binding with these samples was very significant (Figure 5). A two-binding-site process remains until the tertiary structure of the DNA is present. The binding parameters are not significantly affected (Table II) after a treatment with 0.01 M sodium deoxycholate. After a treatment with 0.05 M deoxycholate where the tertiary structure is fully destroyed, only one type of binding sites remain whose pa-

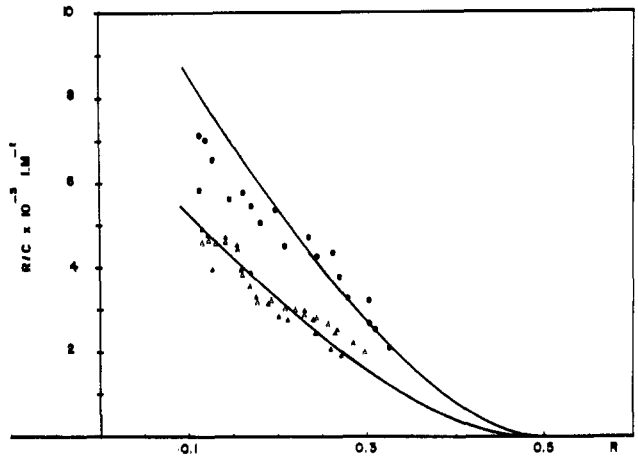

FIGURE 4: Binding isotherms of EB to reconstituted complexes between histone H_1 and DNA for varying PROT/DNA ratios—(●) 0.1; (▲) 0.45; (△) 0.7.

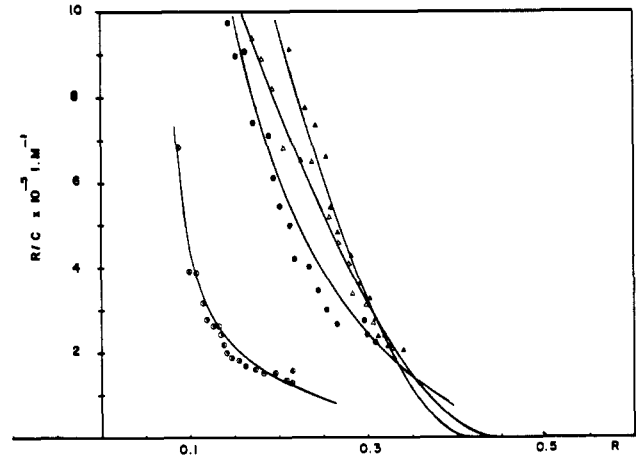

FIGURE 5: Effect of sodium deoxycholate extraction upon EB binding isotherms. The concentrations used were: 0.01 M (⊙); 0.03 M (●); 0.08 M (▲); 0.1 M (△).

rameters are of the order of magnitude of those of pure DNA. After a treatment with 0.03 M, the binding pattern is very peculiar: 40% of the DNA has a binding affinity which is four times higher than that of pure DNA. The remaining 60% presents a behavior roughly similar to that of the second type of binding sites in the native chromatin. These results strongly suggest that, in addition to histone H_1, the tertiary structure of the DNA is essential to determine the two-binding-site process.

Discussion

The use of chemical probes has been widely developed to estimate the accessibility of DNA in chromatin, either with biochemical probes such as DNase (Clark and Felsenfeld, 1974) or chemical probes such as polyamines (Itzhaki, 1974) or fluorescent labels (Lawrence and Louis, 1972; Angerer and Moudrianakis, 1974; Angerer et al., 1974; Lurquin and Seligy, 1972; Bontemps and Fredericq, 1974).

Ethidium bromide, particularly, is very useful in determining the accessibility of DNA in chromatin and partially depleted chromatin. The presence of two types of highly fluorescent sites in chromatin has been described simultaneously by Lawrence and Louis (1974) and Angerer et al. (1974). These latter authors have studied in detail the physical nature of these sites and found a more clustered state for the sites of chromatin as compared with those of DNA. They clearly demonstrated that the heterogeneity of the sites could not be attributed to a variation of the quantum yield of the dye with the amount of bound dye. However, a slight decrease with r of the quantum yield of fluorescence of bound ethidium bromide was observed (Bontemps and Fredericq, 1974). They noted indeed a larger decrease with DNP than with DNA. When these data are taken into account, the error on r determination does not exceed 10% at low values of r and could not explain at all the large difference between DNA and DNP binding isotherms.

In the present study we have tried to give a more quantitative description of the binding properties of chromatin, especially in reevaluating the binding model used to calculate the binding parameters. The best fit method used here gives more accurate values for the binding parameters, especially in describing a two-binding-site process. The values found for the binding parameters make possible quantitative estimation of the influence of various chromatin components upon the binding properties of the DNA. Since we assume that the only mechanism of EB binding is described by the excluded site model of Crothers (1968), and indeed we have verified that all our binding curves fit with $n' = 2$ in eq 3, the only parameters allowed to vary are the binding constant and the number of available binding sites.

Variation of the former may be indicative of change in the free energy of the binding process. This may happen with changes either in the electrostatic potential of the binding site, i.e., under charge neutralization, or in the entropic factor, i.e., conformational change of the site as it has been proved to occur in twisted circular DNA (Bauer and Vinograd, 1968).

We shall assume that decreasing the number of available binding sites may be induced by a topological hindrance of the intercalation process. Such a mechanism may occur for example if some sites are occupied by some amino acid residues of histone molecules, or if the unwinding mechanism necessary for intercalation is prevented by strong enough histone–DNA interaction.

With respect to these preliminary remarks, we can now examine the binding properties of EB by chromatin. Two points appear to deserve a thorough discussion.

(1) The number of low affinity binding sites in native chromatin as well as in reconstituted or 0.01 M deoxycholate-treated chromatin is constant and practically equal to the total number of sites found in partially histone-depleted chromatin (see Tables I and II).

In these five types of material, 80% of the DNA is accessible to intercalative drugs since EB gives rise to enhancement of fluorescence only when intercalated in a double-stranded helical DNA. The accessibility of the DNA inside chromatin has been the field of much controversy (Clark and Felsenfeld, 1971; Itzhaki, 1970; Mirsky, 1971; Schmidt et al., 1972) particularly when the probe used to estimate accessibility was DNA hydrolysis by either pancreatic nuclease or staphylococcal DNase. It seems that potentially up to 95% of the DNA can be digested by pancreatic nuclease while the highest amount of solubilized DNA is about 50% of the initial chromatin DNA when staphylococcal nuclease is used (Clark and Felsenfeld, 1974). The variable acid solubility of the product has been attributed to differences in the rate of aggregation of the released proteins onto the DNA (Itzhaki, 1974).

More concordance is reached when external probes such as polylysine or divalent cations are used with these compounds. Forty percent of the DNA phosphate groups are accessible for binding (Clark and Felsenfeld, 1974; Itzhaki, 1970, 1974). Ethidium bromide used here, according to the model adopted

to calculate the binding parameters, can be considered as an internal probe to DNA. The main advantage of this probe over nuclease is that it does not need to destroy the DNA duplex to operate and then leaves more intact the native configuration of chromatin. However, this last point was recently questioned (Fenske et al., 1975). It seems that the DNA unwinding induced by EB intercalation loosens enough the histone–DNA interaction to observe a release of histones in 0.1 M ammonium sulfate. What can be retained anyway from our data is the large accessibility of the DNA to EB or in other words the possibility to unwind a large amount of the double-stranded helix inside the chromatin. There is now a general agreement on the organization of the major parts of the chromatin (or H_1-depleted chromatin) into "nucleosomes" or "ν bodies" (Olins and Olins, 1974; Noll, 1974; Van Holde et al., 1974; Oudet et al., 1975). It is thus impossible to restrict EB intercalation to regions of free DNA, and DNA unwinding can occur in histone-covered regions.

The blocking of 20% of the DNA molecule is therefore indicative of very strong histone–DNA interactions, preventing internal movements of some parts of the DNA molecule. After removal in 1 M NaCl, of the major part of histones H_{2A} and H_{2B}, the presence of 80 to 90% of H_3 and H_4 still bound to DNA suffices to maintain 18% of the DNA unaccessible to EB. When all of the histones are removed, about 6% of the DNA remains unaccessible, a property which could be related to the presence of nonhistone residual proteins bound to the DNA. We can therefore assume that roughly 14% of the DNA into the chromatin is interacting with histones H_3 and H_4 in such a way that EB intercalation can occur no longer. One could speculate about the role played by the tetrameric units, as described by Kornberg and Thomas, which could be similar to a center of nucleation in which the DNA is maintained in a structure unable to be opened to intercalative drugs. Two explanations can be suggested for such a behavior: (i) electrostatic interactions are large enough to prevent an increase of the distance between two adjacent phosphate groups; (ii) the conformation of the DNA inside this nucleation center is not energetically favorable to an intercalation process.

(2) Histone H_1 has been found to have a very important property in conferring a high binding constant upon a small amount of the DNA in native chromatin. Since an electrostatic factor would have an opposite effect on the binding properties of DNA, one must refer to a change of the entropic factor in the free energy of the binding reaction of EB to DNA. And, indeed, the tertiary structure of DNA was essential to maintain this state. As soon as it is destroyed by complete removal of one of the histones implicated in the octamer structure, the strong binding sites disappear. On the opposite the only effect of binding H_1 to purified DNA was a higher decrease of the binding constant than in the case of the other histones. Such a property could be related, at least qualitatively, to the net positive charge of the H_1 molecule which is by far the highest among all of the histones. In any case, however, H_1 alone was not able to induce a two-site binding process similar to that of chromatin. The tertiary structure of DNA may occur in chromatin under several conditions. Gourevitch et al. (1974) and Crick and Klug (1975) proposed two different ways to generate kinks in DNA and to allow it to bend in tertiary turns. Bending may be imposed by histone interaction with DNA, creating physical constraints in the remaining DNA. Such physical constraints have been found in covalently linked DNA (Bauer and Vinograd, 1968) in which they favor EB intercalation and, more recently (Germond et al., 1975), they have been proved to be generated by histones interaction with relaxed SV40 DNA. Histone H_1 may then have an additional effect upon binding to the tertiary structured DNA. Recently the extent of interaction between this histone and superhelical DNA was found to increase with increasing superhelicity (Vogel and Singer, 1975). From this study, histone H_1 was found to be most likely bound to the superhelical turns themselves.

The effect of binding histone H_1 on such a structure may be of two kinds, either generating a quaternary structure of the DNA or creating additional constraints by itself. In both cases it may generate localized zones in DNA which have accumulated the free energy of the process. This energy is available for binding ethidium bromide and more generally for all kinds of binding involving unwinding processes of the DNA. It is of course tempting to compare the 13% of high-affinity sites with the 14% of sites unaccessible to EB in H_1-depleted chromatin and, therefore, to imagine a release of the hindrance due to H_3 and H_4, consecutive to their interaction with H_1, giving rise to a fully accessible DNA.

In our present state of knowledge there is no reason to do such a comparison and we can only say that the high binding constant of EB is suggestive of a very peculiar conformational state which may reflect specific functional activity.

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Removal of Histone H1 Exposes a Fifty Base Pair DNA Segment between Nucleosomes†

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ABSTRACT: Micrococcal nuclease has been used to prepare chromatin from HeLa cells and to probe the structure of HeLa chromatin under various ionic conditions and after the removal of chromatin proteins by salt extraction. The results suggest that (1) HeLa chromatin DNA exists as 150–160 base pair beads interspersed with 40–50 base pair bridges; (2) the bead and bridge conformation exists at physiologic salt concentrations; and (3) removal of histone H1 renders the 40–50 base pair bridge, but not the 150–160 base pair bead, more nuclease susceptible.

Morphological and biochemical evidence strongly supports the concept that chromatin has a repetitive structure, consisting of subunits composed of an octomeric core of the four small histones surrounded by a segment of the DNA fiber (reviewed by Felsenfeld, 1975). There is some disagreement as to whether all, or only a portion, of the DNA of the chromatin subunit is in close apposition to the histone core. Some investigators suggest that all the DNA of the chromatin subunit strongly interacts with the histone core to form a nucleoprotein "bead" (Noll et al., 1975; Finch et al., 1975); others suggest that only a portion of the DNA is in the "bead" conformation and that the remaining DNA, connecting neighboring subunits, is in a more extended and/or protein-free conformation, generating an overall "bead and bridge" structure (Olins and Olins, 1974; Shaw et al., 1974; Oudet et al., 1975; Langmore and Wooley, 1975; Simpson and Whitlock, 1976; Shaw et al., 1976; Woodcock et al., 1976). Electron microscopic observations (Griffith, 1975) suggest that, at low ionic strength (I = 0.015), a bead and bridge structure might exist but, at higher salt concentrations (I = 0.15), the beads become more closely apposed. We have shown previously that, at low ionic strength,

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