Relaxation of Chromatin Structure by Ethidium Bromide Binding: Determined by Viscometry and Histone Dissociation Studies†

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ABSTRACT: The effects of ethidium bromide intercalation on chromatin structure were monitored by viscometry and analysis of histone dissociation. Investigation of the NaCl concentration dependence of chromatin viscosity showed that the reduced viscosity (η_{red}*) was very low up to 0.4 M NaCl and increased gradually when the salt concentration was raised further. In chromatin intercalated by ethidium bromide, η_{red}* was not significantly different at low salt concentrations (up to 0.2 M NaCl). However, when the salt concentration was raised further, the viscosity response curve increased sharply to reach viscosities about 4–5 times higher than those for nonintercalated chromatin. The increase in viscosity was proportional to the increase in fluorescence intensity, when the ratio of ethidium bromide to DNA nucleotide was raised. The transition of intercalated chromatin into the relaxed form was reversible, dependent on the nature of the electrolyte and cooperative, as indicated by the small increase in salt concentration required to obtain chromatin relaxation. Investigation of the NaCl concentration dependence of histone dissociation revealed that total histones and each individual histone fraction were released from intercalated chromatin at much reduced NaCl concentrations. The midpoints of the dissociation curves of the individual histones ranged from 0.30 to 0.45 M NaCl and fell within the same range where the drastic viscosity change occurred. These results indicate that intercalation of ethidium bromide labilizes chromatin structure to relaxation by moderately elevated salt concentrations. It is suggested that the labilization is caused by changes in the DNA helix conformation due to dye intercalation decreasing the stability of histone–DNA interactions.

A recent model envisages chromatin as organized discontinuously in disk-shaped subunits arranged like beads on a string (Olins and Olins, 1974; Kornberg, 1974; Langmore and Wooley, 1975). In each subunit the DNA is supposedly wound on the outside of a core of assembled histones (Noll, 1974; Baldwin et al., 1975; Pardon et al., 1975). The histone core contains four histone fractions (H2a, H2b, H3, and H4)[1] in a defined molar relationship, while a fifth histone fraction (H1) is located at the subunit periphery (Varshavsky et al., 1976) and has been implicated in participation of a higher order chromatin structure (Bradbury et al., 1975).

The asymmetric distribution of basic and apolar residues in histone fractions H2a, H2b, H3, and H4 results in the formation of a basic N-terminal segment and of an apolar C-terminal segment. The basic histone segments have been suggested to be arranged on the outside of the histone core and to be the primary site of interaction with the DNA, while the apolar histone segments have been implicated in the conformation and the assembly of the histones (Baldwin et al., 1975). This suggests that apolar interactions play an important role in chromatin subunit structure. Recently, organic compounds less polar than water have been found to increase RNA synthesis by added RNA polymerase on chromatin templates (Strätling, 1976). This effect is suggested to be mediated by a weakening of apolar histone interactions, which will dampen the electrostatic interactions between DNA and histones and release the DNA from the constraints imposed by histones.

It is also conceivable that histone–DNA interactions in chromatin are weakened by alterations of the DNA secondary structure. A conformational change of the DNA by complex formation with the intercalative dye ethidium bromide was used to test this suggestion. Intercalation of this dye into the DNA helix changes the rotation angle between adjacent base pairs, and unwinds the right-handed Watson-Crick helix (Waring, 1966). This results in pronounced changes of the physical properties of the DNA, such as a lengthening of the helix and a decrease in helix flexibility (Cohen and Eisenberg, 1968). Chromatin has been reported to have a restricted capacity to intercalate ethidium bromide as compared to deproteinized DNA (Angerer and Moudrianakis, 1972). Since the nature of interaction between DNA and histones in chromatin is mainly that of salt linkages, the sensitivity of chromatin structure to changes in ionic strength is readily apparent. When the salt concentration is raised, histones are dissociated from DNA in three distinct steps (Ohlenbusch et al., 1967; Spelsberg and Hnilica, 1971). Concomitantly with the release of histones, the viscosity of chromatin should increase and approach that of deproteinized DNA. Thus, a change in chromatin conformation may be monitored by viscometry and histone dissociation. These two methods were used in the present study to investigate the effects of ethidium bromide binding on chromatin relaxation.

Materials and Methods

Reagents and Chemical Determinations. Ethidium bromide was purchased from Sigma. All other chemicals were of reagent grade. Individual histone fractions from hen oviduct were prepared according to the method of Oliver et al. (1972) and were observed to be electrophoretically pure. Ethidium bromide concentrations were determined spectrophotometrically assuming an extinction coefficient of 4800 M^{-1} cm^{-1} at 460 nm (Waring, 1965). Chromatin concentrations are expressed in terms of DNA concentrations estimated from measurements

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[1] The histone nomenclature used in this paper was agreed upon at the Ciba Foundation Symposium, 28th (Bradbury, 1975): histone H1 = I = f1; histone H2a = IIb1 = f2a2; histone H2b = IIb2 = f2b; histone H3 = III = f3; histone H4 = IV = f2a1.

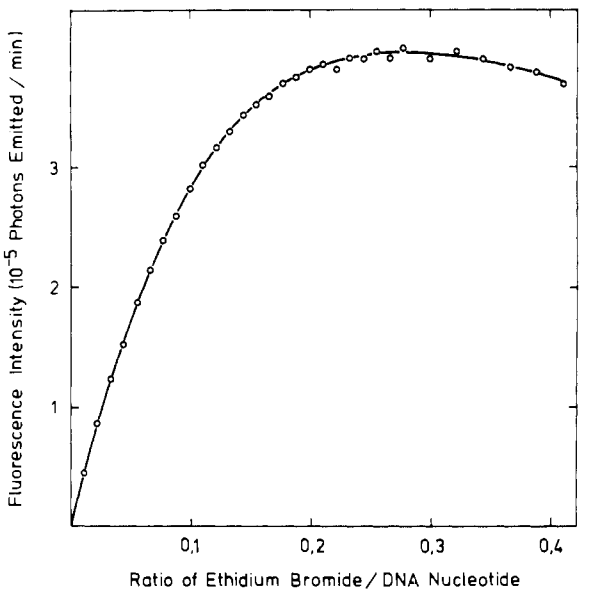

FIGURE 1: The fluorescence enhancement of ethidium bromide bound to oviduct chromatin.

of absorbance at 260 nm. These calculations used 6760 M^{-1} cm^{-1} as the molar extinction coefficient of chromatin at 25 °C in dilutions containing 1% sodium dodecyl sulfate (Johnson et al., 1972) and 0.97 as the fraction of the chromatin absorbance due to DNA.

Chromatin Preparation. Chromatin was prepared from oviduct and liver of laying hens (HNI), spleen and liver of 300-g male rats (Wistar), and calf thymus obtained from the local slaughter house. Nuclei were isolated from these organs according to Chevaillier and Philippe (1973). The pelleted nuclei were lysed by extensive homogenization in 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA,[2] and the resultant chromatin preparation was purified by sedimentation through 1.7 M sucrose in 2.5 mM Tris-HCl, pH 7.2, 24 mM EDTA, 10 mM NaCl (Bhorjee and Pederson, 1973). The purified chromatin pellet was resuspended in 20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA. The RNA to DNA ratio of this preparation was 0.03:1.00 or less. For viscometric and histone dissociation measurements, aliquots of the chromatin suspension were made 0.15 M with respect to NaCl and centrifuged at 13 000 rpm for 20 min in the Sorvall-HB4 rotor. The pelleted chromatin samples were resuspended in 20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA containing NaCl and ethidium bromide at concentrations as indicated under Results.

Fluorescence Studies. Fluorescence measurements were performed in a spectrofluorometer assembled from a number of components, as follows. Samples were illuminated with light from a tungsten lamp powered by a Gilford Instrument Model 240 power supply. A Jarrell-Ash excitation monochromator, Model 82-410, was set at a wavelength of 520 nm and had a linear band-pass of 10 μm and the emission monochromator of the same model was set at 600 nm and had a slit width of 0.5 nm. The intensity of the emitted light was monitored with a sensitive photon counting system consisting of an EMI9635QB bialkali photomultiplier, a S.S.R. Instruments Co. Model 1110 digital synchronous computer, a S.S.R. Instruments Co. Model 1120 amplifier/discriminator, and a S.S.R. Model 1106 power supply. Measurements were made at right angle in a quartz cuvette at 22 °C. Aliquots (5 μl) of an ethidium bromide solution (10^{-3} M) were added to chromatin solutions at concentrations of 1.5×10^{-4} M DNA nucleotide in 20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA.

Analysis of Histone Dissociation. Chromatin suspensions at DNA concentrations of 50 μg/ml were homogenized in the desired salt and dye solutions by whirl mixing for 15 min at 4 °C. Separation of dissociated from DNA-bound histones was performed by centrifugation in an SW40 rotor for 12 h at 40 000 rpm and 0 °C. The supernatant fraction was dialyzed against repeated changes of 0.1 M acetic acid for 48 h and lyophilized. The pelleted fraction was rinsed with distilled water. Histones were extracted from the supernatant and pellet fraction twice with 0.4 N H_2SO_4, and quantitatively determined with the Lowry procedure using calf thymus histones as standard (Lowry et al., 1951). Histone composition was analyzed by polyacrylamide gel electrophoresis in the presence of 2.5 M urea at pH 3.2 (Panyim and Chalkley, 1969). Gels were stained with Naphthol blue black, destained by diffusion, and scanned on a Gilford spectrophotometer; the areas under the peaks of the resultant curves were measured. In order to construct reference curves for quantitation of the amount of histone represented by a single band, known amounts of each histone fraction were loaded on gels, electrophoresed, and scanned under the same conditions as used for the histone samples to be analyzed. Best resolutions of the closely spaced bands for histones H3, H2b, and H2a were obtained by scanning the gels first at 670 nm, a wavelength at which the extinction coefficients of the dye complexes with histones H3 and H2a were relatively high compared to that with histone H2b. The opposite was found at 570 nm, where the histone H2b–dye complex was optimally recorded. The histone dissociation curves in Figure 8 were obtained by correcting the quantitated amounts of histone present in each band of Figure 7 for the differences in the amount of histone sample applied to the gel and expressing this value as the percentage of total (dissociated and bound) amount of each histone fraction.

Viscosity Determinations. Viscosity measurements were performed at 24.2 °C using a micro Ubbelohde viscometer from Schott, Mainz. The temperature was controlled to ±0.01 °C. As chromatin aggregated differentially at various NaCl concentrations, it is important to note that the values for the viscosity parameters are not interpretable in terms of classical hydrodynamic theory. To emphasize this fact we have denoted the parameters with asterisks as η_{sp}* and η_{red}*, where η_{sp}* is the specific viscosity, and η_{red}* the reduced viscosity (η_{red}* = η_{sp}*/[DNA]).

Results

Fluorescence Enhancement of Bound Ethidium Bromide. To determine the range of ethidium bromide concentration, at which dye intercalation might optimally relax chromatin structure, the enhancement of fluorescence of ethidium bromide bound to hen oviduct chromatin was measured as a function of the ethidium bromide concentration. Figure 1 shows that the relative fluorescence intensity of bound ethidium bromide reached a maximum at a dye to DNA nucleotide ratio of about 0.2–0.3. This fluorescence enhancement has been shown to result from the occupation of the strongly fluorescent binding sites (Angerer and Moudrianakis, 1972). At higher dye to DNA nucleotide ratios, the fluorescence intensity declined gradually. This decrease has also been observed by Angerer and Moudrianakis (1972), and may represent either a conformational change of the helical structure by dye molecules bound in excess or an energy transfer between dye molecules (Angerer and Moudrianakis, 1972).

[2] Abbreviation used: EDTA, ethylenediaminetetraacetic acid.

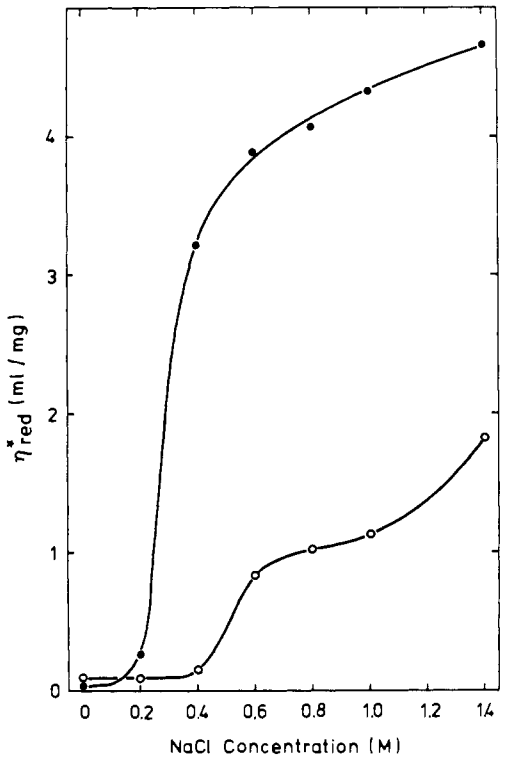

FIGURE 2: Effect of ethidium bromide on the NaCl concentration dependence of η_{red}*. η_{red}* was determined at various NaCl concentrations using untreated chromatin (O—O), and ethidium bromide intercalated chromatin (●—●). The dye to DNA nucleotide ratio was 0.35.

Viscosity Measurements. In order to determine the influence of ethidium bromide binding on the salt concentration dependent relaxation of chromatin, hen oviduct chromatin was incubated at various NaCl concentrations in the absence of ethidium bromide and at a dye to DNA nucleotide ratio of 0.20–0.35. The open circles in Figure 2 show that the reduced viscosity (η_{red}*) of untreated chromatin was nearly indistinguishable from that of the buffer up to 0.4 M NaCl. Above this salt concentration, η_{red}* increased gradually. Aggregation of chromatin was maximal at 0.2 M NaCl (see also Strätling et al., 1976). Also, at zero and 0.4 M NaCl, oviduct chromatin displayed significant aggregation. Consequently, as pointed out already under Materials and Methods, the data cannot be interpreted in terms of classical hydrodynamic theory.

The shape of the viscosity response curve for oviduct chromatin intercalated by ethidium bromide differed significantly from that of nonintercalated chromatin. The values of η_{red}* at zero and 0.2 M NaCl were similar to those of untreated chromatin. However, when the salt concentration was raised from 0.2 to 0.4 M NaCl, viscosity increased drastically to reach values about 4–5 times higher than those of nonintercalated chromatin. Such a drastic change with a very small variation of the external conditions is characteristic of a cooperative transition. When the viscosity of intercalated chromatin was measured as a function of the ratio of ethidium bromide to DNA nucleotide concentration, η_{red}* increased almost linearly up to a ratio of 0.2 and reached a plateau at ratios of 0.2–0.3 (Figure 3). It is apparent that the shape of the viscosity response curve was very similar to that of the fluorescence response curve of oviduct chromatin (see Figure 1), indicating that the viscosity increase was proportional to the frequency of sites intercalated by ethidium bromide.

Values of η_{sp}* for chromatin relaxed by ethidium bromide binding at 0.8 M NaCl were determined at various chromatin

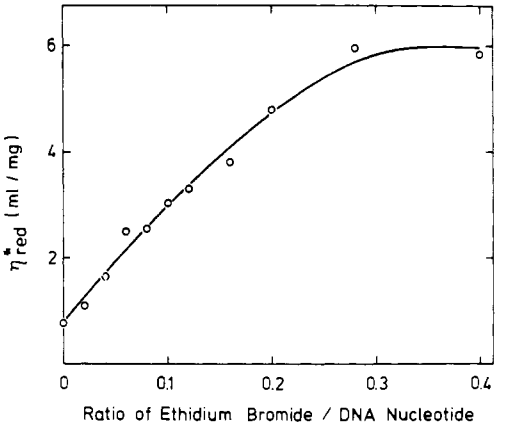

FIGURE 3: Viscometric dependence of η_{red}* of ethidium bromide relaxed chromatin on the dye to DNA nucleotide ratio.

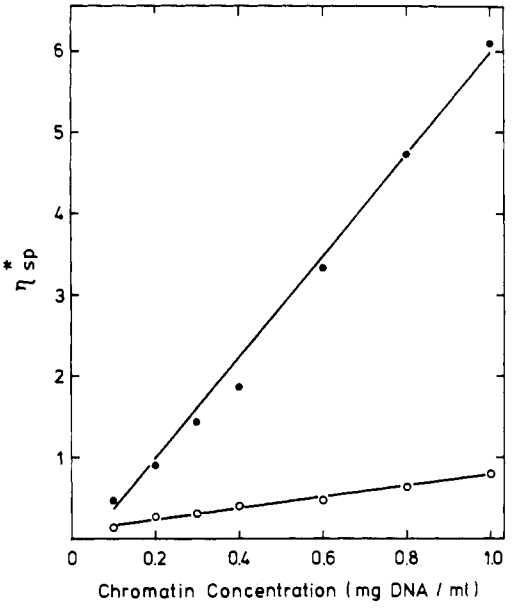

FIGURE 4: Effect of chromatin conformation on the concentration dependence of η_{sp}*. η_{sp}* was determined at various DNA concentrations using chromatin partially dissociated by 0.8 M NaCl (O—O), and chromatin relaxed by treatment with ethidium bromide at a dye to DNA nucleotide ratio of 0.35 in the presence of 0.8 M NaCl (●—●).

concentrations along with similar determinations for chromatin partially dissociated by 0.8 M NaCl. These values are plotted against the DNA concentrations in Figure 4. The slope of the line corresponding to η_{red}*(ethidium bromide, 0.8 M NaCl) was about nine times higher than that of η_{red}* for chromatin partially relaxed by 0.8 M NaCl.

Varying the electrolyte produced the same transition, but at a different salt concentration. Figure 5 shows the viscosity response curves as a function of the molar concentrations of NaCl, LiCl, and $(NH_4)_2SO_4$. At a dye to DNA nucleotide ratio of 0.2 the midpoints of the transitions were 0.44 M NaCl, 0.36 M LiCl, and 0.15 M $(NH_4)_2SO_4$. As seen already in Figure 2, the drastic viscosity increases were produced by only very small changes of the salt concentration. The transition was completely reversible, when the chromatin solution was dialyzed to low salt concentration while maintaining the ethidium bromide concentration.

To demonstrate that the viscosity increase due to ethidium bromide intercalation is a general property of chromatin, preparations from four sources other than hen oviduct were

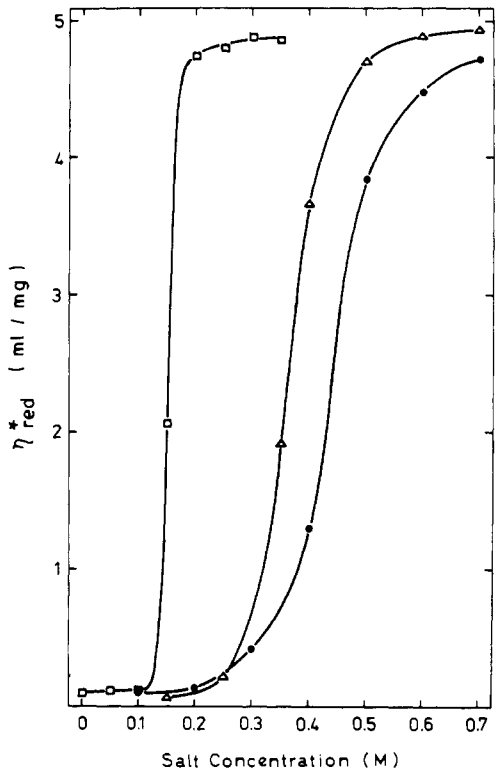

FIGURE 5: Influence of the electrolyte nature on the salt concentration dependence of ethidium bromide induced chromatin relaxation. (●—●) NaCl; (△—△) LiCl; (□—□) $(NH_4)_2SO_4$. The dye to DNA nucleotide ratio was 0.20.

TABLE I: Effect of Ethidium Bromide on η_{red}* Using Chromatin from Various Sources.[a]

Chromatin Source	η_{red}* (0.6 M NaCl) (ml/mg)	η_{red}* (Ethidium Bromide, 0.6 M NaCl) (ml/mg)
Hen oviduct	0.86	3.9
Hen liver	0.66	3.6
Rat liver	0.69	4.0
Calf thymus	0.73	4.1
Rat spleen	0.78	4.2

[a] The ethidium bromide to DNA nucleotide ratio was 0.35.

incubated with the intercalative dye (Table I). These chromatin preparations showed marked viscosity increases, after ethidium bromide binding, similar to the one seen with oviduct chromatin. In addition to ethidium bromide, binding of several other intercalative dyes was shown to labilize chromatin structure to relaxation at 0.6 M NaCl. Table II shows that the increase in viscosity obtained with these dyes was as great as the one seen after ethidium bromide binding. Binding of the nonintercalative dye quinacrine mustard (Michelson et al., 1972) was found to be ineffective in increasing η_{red}* of chromatin, indicating that the capacity to labilize chromatin structure to ionic strength dependent relaxation is strictly correlated with the ability to intercalate into the DNA helix.

Dissociation of Histones. To determine whether the marked viscosity increase observed in intercalated chromatin is associated with an enhanced dissociation of histones from DNA, the effect of ethidium bromide on the salt dependence of histone release was investigated. Dissociated histones were separated from bound histones by high-speed centrifugation, followed by quantitation of total and individual histones in the supernatant and the pelleted nucleohistone fraction. In the absence of ethidium bromide, dissociation of total histones from oviduct chromatin started at 0.4 M NaCl and gradually increased to reach a value of 78% at 1.4 M NaCl (Figure 6). Significantly, the profile of the dissociation curve exhibited a shoulder between 0.9–1.0 M NaCl, similar to the one seen in the viscosity response curve of untreated chromatin (see the open circles in Figure 2). It will be shown later (in Figure 8) that the dissociation of the lysine-rich histones reached completion at about 0.9 M NaCl, whereas the arginine-rich histones did not start to be released at this ionic strength. Therefore, the transient plateau in the viscosity profile and the dissociation response curve for total histones can be explained by the differential dissociation of histone classes at characteristic NaCl concentrations.

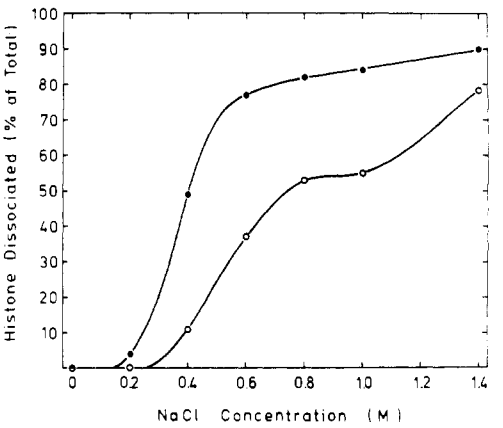

FIGURE 6: Effect of ethidium bromide on the NaCl concentration dependence of histone dissociation from DNA. The fractional amount of dissociated histones was determined at various NaCl concentrations using untreated chromatin (○—○) and ethidium bromide relaxed chromatin (●—●).

TABLE II: Effect of Various Complexing Agents on η_{red}* of Oviduct Chromatin.[a]

Complexing Dye	η_{red}* (dye, 0.6 M NaCl) (ml/mg)
None	0.83
Ethidium bromide	3.08
Acridine orange	3.18
Proflavine	3.04
Actinomycin D	2.76
Quinacrine mustard	0.76

[a] The dye to DNA nucleotide ratio was 0.20.

The results from untreated chromatin were compared with the dissociation response curve obtained in the presence of ethidium bromide at a dye to DNA nucleotide ratio of 0.35. When the salt concentration was raised from 0.2 to 0.6 M NaCl, dissociation of histones rapidly increased to reach a relative plateau of about 80% (Figure 6). The markedly enhanced dissociation of histones in intercalated chromatin occurred within the same narrow range of ionic strength increase as the drastic viscosity change (see Figures 2 and 5). At a dye to DNA nucleotide ratio of 0.2, ethidium bromide was about as effective as at the higher ratio (0.35) in promoting the release of total histones when the NaCl concentration was raised from 0.2 to 0.6 M. The high tendency of histones to form ag-

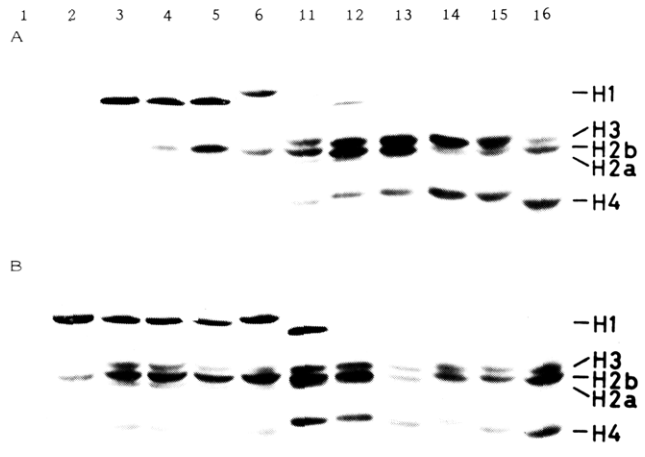

FIGURE 7: Gel electrophoresis of dissociated and bound histones. (A) Acrylamide–urea gel electrophoresis patterns of histones dissociated from oviduct chromatin with (1) 0.2, (2) 0.4, (3) 0.6, (4) 0.8, (5) 1.0, and (6) 1.4 M NaCl, and histones pelleting with DNA after extraction with (11) 0.2, (12) 0.4, (13) 0.6, (14) 0.8, (15) 1.0, and (16) 1.4 M NaCl. (B) Acrylamide–urea gel electrophoresis patterns of histones dissociated from oviduct chromatin in the presence of ethidium bromide at a dye to DNA nucleotide ratio of 0.35 with (1) 0.2, (2) 0.4, (3) 0.6, (4) 0.8, (5) 1.0, and (6) 1.4 M NaCl, and histones pelleting with DNA after extraction with (11) 0.2, (12) 0.4, (13) 0.6, (14) 0.8, (15) 1.0, and (16) 1.4 M NaCl.

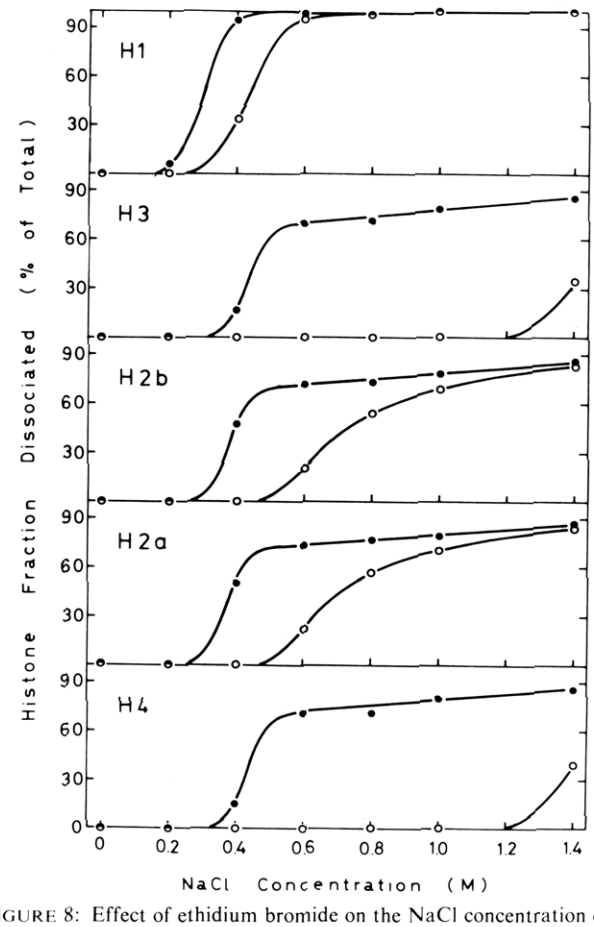

FIGURE 8: Effect of ethidium bromide on the NaCl concentration dependence of the dissociation of individual histone fractions from DNA. From the densitometer scans of the gels shown in Figure 7 and from the fractional amounts of histones loaded on the gels, the dissociation curves of histone fractions H1, H2a, H2b, H3, and H4 were constructed for untreated chromatin (O—O) and chromatin relaxed by ethidium bromide treatment at a dye to DNA nucleotide ratio of 0.35 (●—●).

gregates at moderate and high ionic strength (Edwards and Shooter, 1969) gives a reasorable explanation why the curve for apparent histone dissociation did not reach 100%. Dissociated, but aggregated histones would be found after high-speed centrifugation in the pelleted nucleohistone fraction. Therefore, the given percentages of histone dissociation should be minimal estimates. In some experiments proteolytic action was controlled by including the protease inhibitor phenylmethylsulfonyl fluoride during tissue disruption and further nuclei and chromatin preparation. These experiments showed results identical to those obtained with chromatin purified in the absence of the inhibitor. In addition, the polyacrylamide gels shown in Figure 7A,B give no indication of major histone degradation by proteases. These results allow the conclusion that proteolytic degradation of histones does not contribute to chromatin relaxation and histone dissociation in the presence and absence of ethidium bromide.

To investigate the effect of ethidium bromide binding on the dissociation of the individual histone fractions, the gel electrophoresis patterns of histones dissociated at various NaCl concentrations were compared with the patterns of histones remaining bound to DNA. Figure 7A shows that in the absence of ethidium bromide histone H1 was dissociated between 0.4 and 0.6 M NaCl. The histone fractions H2a and H2b were extracted between 0.6 and 1.0 M NaCl, while the histones H3 and H4 started to dissociate at 1.4 M NaCl. A similar comparison is made between the gel patterns of histones dissociated in the presence of ethidium bromide and the patterns of histones remaining with DNA under these conditions (Figure 7B). The markedly enhanced dissociation of histones after ethidium bromide binding is readily seen. All individual histone fractions started to be extracted at much lower NaCl concentrations: histone H1 at about 0.2 M NaCl and histones H2a, H2b, H3, and H4 between 0.2 and 0.4. The dissociation curves in Figure 8 compare the release of each histone fraction from nonintercalated DNA to that from DNA intercalated by ethidium bromide. Dye binding shifted the dissociation curve for histone H1 to a position with half-maximal release at about 0.30 M NaCl. A comparison of the dissociation curves obtained for histone fractions H2a and H2b, in the presence and absence of ethidium bromide, revealed that, after dye intercalation, the NaCl concentration required to release these histones was decreased to an even greater extent: half-maximal dissociation occurred between 0.35 and 0.40 M NaCl. The most drastic reduction of dissociating NaCl concentration, after ethidium bromide binding, was seen for histone fractions H3 and H4. The midpoints of the dissociation curves of these histones were shifted from about 1.4 to about 0.40–0.45 M NaCl after binding of the intercalative dye. It is evident that all histone fractions dissociated from intercalated chromatin over a very small increase of the NaCl concentration with midpoints of the dissociation curves between 0.30 and 0.45 M NaCl. Within this very same narrow range of NaCl concentration, the drastic enhancement of viscosity was seen (Figures 2 and 5). Although the histone dissociation curves for intercalated chromatin are closely clustered, it is possible to see that histone H1 dissociated first, histones H2a and H2b at somewhat higher salt concentrations, followed closely by histones H3 and H4. A corresponding differential effect of increasing NaCl concentration was seen on histone dissociation from nonintercalated chromatin. However, in the absence of ethidium bromide, the NaCl concentration range within which dissociation occurred was much more widespread with midpoints of the dissociation curves from about 0.45 to about 1.4 M NaCl.

The dissociation curves for histone H1 reached 100% dissociation, but this was not obtained for the other histone fractions. This observation is related to the earlier finding that the apparent dissociation curve for total histones did not reach 100% under the conditions used (see Figure 6). Comparisons made in 0.1 M NaCl (Edwards and Shooter, 1969) indicated that histone H1 does not aggregate at this ionic strength, whereas the other histone fractions form large aggregates. Therefore, the plateau at about 80% dissociation reached by the dissociation curves of histones H2a, H2b, H3, and H4 can best be explained by salt-induced self-aggregation.

Discussion

The present work has shown that a number of dyes, known to bind to duplex DNA by intercalation, labilizes chromatin structure to salt concentration dependent relaxation and histone dissociation. Viscometry is demonstrated to be a very sensitive tool to study the conformational transitions in chromatin. The NaCl concentration dependence of chromatin viscosity appears to reflect several different processes. First, compaction of chromatin displays a characteristic salt dependence with maximal aggregation at 0.2 M NaCl. Above 0.4 M NaCl, however, two other processes mainly affect chromatin viscosity. Ross and Scruggs (1964) and Cohen and Eisenberg (1968) have shown that the viscosity of deproteinized DNA decreases as the salt concentration is raised, probably due to DNA compaction as the salt dampens intramolecular electrostatic repulsive forces. However, viscosity due to complexation of DNA with histones in chromatin would behave the opposite way, since salt also dampens the electrostatic forces between histones and DNA and leads to increased relaxation and increased viscosity. The open circles in Figure 2 show that the reduced viscosity of untreated chromatin was little affected as the salt concentration was raised from 0 to 0.4 M NaCl. Above 0.4 M NaCl, chromatin viscosity increased gradually, indicating that a dissociation phenomenon was being measured above this salt concentration.

The combination of intercalative dyes with deproteinized DNA has been shown to result in a markedly enhanced viscosity due to extension and unwinding of the DNA helix (Lerman, 1961). The closed circles in Figure 2 show that this effect was not observed with intercalated chromatin at low ionic strength (up to 0.2 M NaCl concentration). However, viscosity markedly increased as salt concentration was raised above 0.2 M NaCl. It is apparent that in intercalated chromatin relaxation was obtained at a much reduced salt concentration. As the interactions between DNA and histones are mainly that of salt linkages, which are dampened by an increase in ionic strength, it may be interferred that these forces are weakened by conformational changes of the DNA helix which resulted from ethidium bromide intercalation. The increase in viscosity was proportional to the increase in fluorescence intensity, when the ratio of ethidium bromide to DNA nucleotide concentration was raised. This result indicates that the occupation of the strongly fluorescent binding sites caused chromatin to relax.

Viscosity of chromatin intercalated by ethidium bromide increased sharply over a NaCl concentration range of 0.3–0.5 M. This viscometric result is entirely consistent with parallel measurements of the salt concentration dependent release of histones from DNA, as histone dissociation in intercalated chromatin increased markedly over the same salt concentration range. The characteristically steep slopes of the viscosity and dissociation response curves suggest that the relaxation of chromatin may be highly cooperative. The exact degree of sharpness of the transition will, however, depend on the homogeneity of the system. Histones are composed of five major fractions and Figure 8 indicated that these fractions were released in intercalated chromatin at rather close, but clearly distinct NaCl concentrations. Probably for this reason, the dissociation curve of total histones is somewhat shallower than those of the individual histone fractions. In addition, the viscosity vs. NaCl concentration profile in Figure 5 is suggestive of some heterogeneity. At the ionic strength used in this study, ethidium bromide can interact only very weakly with the histones, which is in contrast to the high affinity of the dye to the DNA helix (Waring, 1965). Therefore, ethidium bromide is unlikely to cause the enhanced dissociation of histones by a direct interaction with the histones, rather than by an indirect effect by intercalating into the double helix.

We propose that the effects of ethidium bromide on chromatin relaxation are related to its intercalation into the DNA helix. Evidence for this assumption is as follows. (1) Destabilization of chromatin structure was specific for DNA intercalators. (2) The increase in the occupation of strongly fluorescent binding sites was directly correlated to the increase in chromatin viscosity as the salt concentration was raised. Intercalation of ethidium bromide into DNA complexed with histones can be expected to cause similar changes of the DNA structure, as in deproteinized DNA, i.e., an increase in the rotation angle between adjacent base pairs and an unwinding of the helix (Waring, 1966). It may be suggested that the resulting pronounced changes of the physical properties of the DNA (Cohen and Eisenberg, 1968) interfere strongly with the histone–DNA interactions. A recently formulated model of chromatin structure may help to give a better understanding of this interference. The model suggests that the DNA in chromatin is wound on the outside of a core of assembled histones (Baldwin et al., 1975; Pardon et al., 1975; Noll, 1974). It may be hypothesized that negative phosphate charges of the wound or kinked helix exactly fit with positive charges of basic amino residues on the histone core. The dye-indueed twisting and lengthening of the helix (Cohen and Eisenberg, 1968) may well cause a gross perturbation of the complementarity in histone and DNA structure. Also, the dye-induced stiffening of the helix (Cohen and Eisenberg, 1968) may prevent the helix from winding around the histone core. A weakening of the interactions between DNA and histones can easily explain the labilization of chromatin to salt-mediated histone dissociation. The association of protein and DNA to form bacteriophage T2 has been shown to be prevented by proflavine (De Mars, 1955). This finding, as well as our relaxation experiments, suggest that intercalation labilizes DNA–protein complexes to ionic strength and prevents assembly of such complexes because it interferes with DNA–protein interactions.

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References

Angerer, L. M., and Moudrianakis, E. N. (1972), *J. Mol. Biol. 63,* 505.

Baldwin, J. P., Bosely, P. G., Bradbury, E. M., and Ibel, K. (1975), *Nature (London) 253,* 245.

Bhorjee, J. S., and Pederson, T. (1973), *Biochemistry 12,* 2766.

Bradbury, E. M. (1975), *Ciba Found. Symp. 28,* 2.
Bradbury, E. M., Danby, S. E., Rattle, H. W. E., and Giancotti, V. (1975), *Eur. J. Biochem. 57,* 97.
Chevaillier, P., and Philippe, M. (1973), *Exp. Cell Res. 82,* 1.
Cohen, G., and Eisenberg, H. (1968), *Biopolymers 6,* 1077.
De Mars, R. I. (1955), *Virology 1,* 83.
Edwards, P. A., and Shooter, K. V. (1969), *Biochem. J. 114,* 227.
Johnson, R. S., Chan, A., and Hanlon, S. (1972), *Biochemistry 11,* 4347.
Kornberg, R. D. (1974), *Science 184,* 868.
Langmore, J. P., and Wooley, J. C. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2691.
Lerman, L. S. (1961), *J. Mol. Biol. 3,* 18.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Michelson, A. M., Monny, C., and Kovoor, A. (1972), *Biochimie 54,* 1129.
Noll, M. (1974), *Nucleic Acids Res. 1,* 1573.
Ohlenbusch, H. H., Olivera, B. M., Tuan, D., and Davidson, N. (1967), *J. Mol. Biol. 25,* 299.
Olins, A. L., and Olins, D. E. (1974), *Science 183,* 330.
Oliver, D., Sommer, K. R., Panyim, S., Spiker, S., and Chalkley, R. (1972), *Biochem. J. 129,* 349.
Panyim, S., and Chalkley, R. (1969), *Arch. Biochem. Biophys. 130,* 337.
Pardon, J. F., Worcester, D. L., Wooley, J. C., Tatchell, K., Van Holde, K. E., and Richards, B. M. (1975), *Nucleic Acids Res. 2,* 2163.
Ross, P. D., and Scruggs, R. L. (1964), *Biopolymers 2,* 79.
Spelsberg, T. C., and Hnilica, L. S. (1971), *Biochim. Biophys. Acta 228,* 202.
Strätling, W. H. (1976), *Nucleic Acids Res. 3,* 1203.
Strätling, W. H., Van, N. T., and O'Malley, B. W. (1976), *Eur. J. Biochem. 66,* 423.
Varshavsky, A. J., Bakayev, V. V., and Georgiev, G. P. (1976), *Nucleic Acids Res. 3,* 477.
Waring, M. J. (1965), *J. Mol. Biol. 13,* 269.
Waring, M. J. (1966), *Biochim. Biophys. Acta 114,* 234.