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Interactions between Arginine-Rich Histones and Deoxyribonucleic Acids. II. Circular Dichroism†

Sharon S. Yu, Hsueh Jei Li,* and Thomas Y. Shih

ABSTRACT: Circular dichroism (CD) was used to investigate the conformations of arginine-rich histones, H3 (III or f3) and H4 (IV or f2a1), and DNA in the complexes prepared by four different methods: (A) NaCl gradient dialysis with urea; (B) NaCl gradient dialysis without urea; (C) direct mixing in 2.5 $\times 10^{-4}$ M EDTA, pH 8.0; and (D) direct mixing in 0.01 M sodium phosphate, pH 7.0. Using the CD spectrum of native chromatin as a criterion to judge the closeness of a complex to its native state, it was observed that a complex made by direct mixing at low ionic strength (methods C and D) is better than the ones made by NaCl gradient dialysis with or without urea (methods A and B). It is explained as a result of lack of ordered secondary structures in histones due to the presence of urea in method A or due to nonspecific aggregation in NaCl without urea (method B). Compared with all the earlier reports in literature on the CD of histone–DNA complexes, the CD spectra of arginine-rich histone–DNA complexes prepared by methods C and D are closest to that of native chromatin both in shape and in amplitude. These results imply (a) that arginine-rich histones play an important role in maintaining the conformation of chromatin and (b) that the binding of these two histones to DNA prepared by methods C and D are close to that in native chromatin. Noticeable variation in conformation of free and bound histone and histone-bound DNA has also been observed in histone H3 with one or two cysteine residues, and in reduced or oxidized state even when the complexes were prepared and examined in the same condition. CD spectra of arginine-rich histones in 0.01 M phosphates, pH 7.0, indicate the presence of α-helix which could be responsible for a favorable binding of the less basic regions of these histones to DNA under this condition as demonstrated by thermal denaturation (Yu, S. S., Li, H. J., and Shih, T. Y. (1976), *Biochemistry,* the preceding paper in this issue). To preserve or generate α-helical structures in histones seems to be a critical step in reconstituting good histone–DNA complexes.

Both histones and DNA are macromolecules. Their interactions inevitably depend upon their secondary, tertiary, and quaternary structures. These structures, in turn, depend upon the solution conditions and the presence of other macromolecules, such as various species of histones, nonhistone proteins, and RNA. The most sensitive tool for investigating the secondary structures of macromolecules is circular dichroism (CD) which has been used extensively in the past decade for the studies of histones (Bradbury et al., 1965; Jirgenson and Hnilica, 1965; Li et al., 1972; D'Anna and Isenberg, 1974a,b,c), DNA (Johnson and Tinoco, 1968; Tunis-Schneider and Maestre, 1970), histone–DNA complexes (Olins, 1969; Fasman et al., 1970; Shih and Fasman, 1971; Li et al., 1971; Adler et al., 1974, 1975; Leffak et al., 1974), and chromatin

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(Shih and Fasman, 1970; Simpson and Sober, 1970; Permogorov et al., 1970; Johnson et al., 1972; Chang and Li, 1974; Hjelm and Huang, 1974; Wilhelm et al., 1974; Hanlon et al., 1974; Li et al., 1975).

CD properties of chromatin are well characterized. The DNA CD (above 250 nm) in chromatin suggests conformational change in DNA due to the binding of histones, while the histone CD (near 220 nm) indicates a large amount of ordered structures in bound histones. Previous CD studies on histone-DNA complexes such as histone H1 (I or f1)-DNA (Olins, 1969; Fasman et al., 1970; Sponar and Firc, 1972), histone H4 (IV or f2a1)-DNA (Wagner, 1970; Shih and Fasman, 1971; Li et al., 1971; Adler et al., 1975), histone H2B (IIb2 or f2b)-DNA (Adler et al., 1974; Leffak et al., 1974), and histone H5 (V or f2c)-DNA (Hwan et al., 1975) put more emphasis on DNA CD and less on histone CD. In these cases, the DNA CD varies greatly depending upon the method of complex formation. In addition, the histone CD in these complexes, in general, is small compared with that in chromatin.

The main objectives for this study are twofold: (a) to find how the CD properties of DNA and histones vary as a function of the method of complex formation, conformation of histones, and histone–histone interactions before binding to DNA; and (b) to find the conditions in which the complexes show physical properties (thermal denaturation and CD) most resembling those in native chromatin.

Materials and Methods

Materials and methods used for this report are essentially identical with those described in an earlier paper (Yu et al., 1976).

Four different methods were used for complex formation: (A) continuous NaCl gradient dialysis with urea (Li and Bonner, 1971), as modified from stepwise gradient dialysis with urea (Bekhor et al., 1969; Huang and Huang, 1969); (B) continuous NaCl gradient dialysis without urea (Huang et al., 1964); (C) direct mixing in 2.5×10^{-4} M EDTA, pH 8.0; and (D) direct mixing in 0.01 M sodium phosphate, pH 7.0. Unless specified otherwise, the final buffer used for CD measurement was 2.5×10^{-4} M EDTA, pH 8.0.

The input ratio of histone to DNA is reported as r (amino acid residues/nucleotide). CD measurements were made on a Jasco spectropolarimeter Model J-20 at room temperature. $\Delta\epsilon = \epsilon_L - \epsilon_R$ is reported where ϵ_L and ϵ_R are respectively molar extinction coefficients of the left- and the right-handed circularly polarized light. The unit for $\Delta\epsilon$ is M^{-1} cm^{-1} where M represents moles/liter in nucleotide for DNA and complexes and in amino acid residues for free and bound histones.

Results

Complexes between DNA and Arginine-Rich Histones Prepared by NaCl Gradient Dialysis. The CD results of calf thymus histone H3-DNA complexes prepared by NaCl gradient dialysis with urea (method A) are shown in Figures 1a and 1b while those by gradient dialysis without urea (method B) in Figures 1c and 1d. As the r value of each complex is raised, there is a greater reduction in the positive CD band near 275 nm and a greater red shift of both the peak and the crossover point of this band. The reduction in amplitude is similar to that seen in chromatin (Shih and Fasman, 1970); the red shift, on the other hand, is much greater.

Complexes of higher r value show a slightly more negative CD below 230 nm which could be attributed to bound histones. The CD contribution from histone H3 in these complexes, however, is much too small compared with those contributed

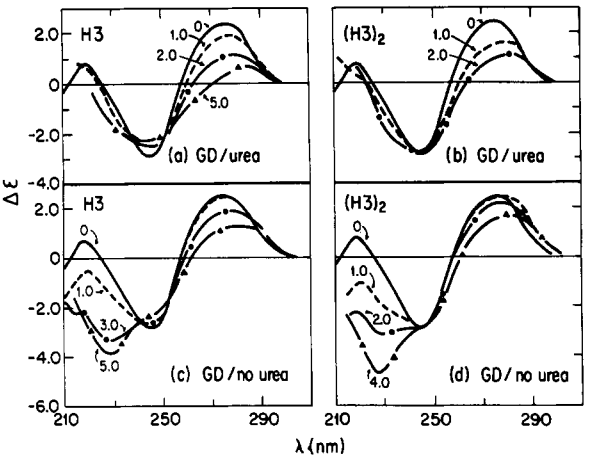

FIGURE 1: Circular dichroism (CD) spectra of calf thymus histone H3-DNA and $(H3)_2$-DNA complexes prepared by NaCl gradient dialysis with urea (GD/urea) and without urea (GD/no urea). r value of each complex is indicated.

by histones in either native chromatin (Shih and Fasman, 1970) or histone H1-deprived chromatin (Li et al., 1975). Nevertheless, the CD spectra of the complexes are more or less the same, whether the reduced histone H3 or histone H3 dimer $(H3)_2$ is used. As far as the secondary structures of both DNA and histones in the complex are concerned, the reduced histone H3 and oxidized histone H3 dimer behave in a similar way.

The results in Figures 1c and 1d show that the complexes prepared by gradient dialysis without urea (method B), in general, have less CD effects on DNA (both of the amplitude and the red shift of the crossover) but possess more negative CD near 220 nm for histones than those complexes prepared by gradient dialysis with urea (method A). Perhaps the presence of urea in the medium (method A) could potentially destroy the ordered structures of histone H3 before or after it is bound to DNA. Again, there is no major difference in the CD results between complexes made with reduced histone H3 and complexes made with oxidized histone H3 dimer.

Using stepwise NaCl gradient dialysis in urea, Shih and Fasman (1971) reported no changes in DNA CD near 275 nm for reconstituted nucleohistone H4 if the final medium was 0.01 M NaF-0.001 M Tris (pH 7.0). The highest r value of their complexes was 2.5. Li et al. (1971) came to a similar conclusion when complexes of nucleohistone H4, with r values of 0 to 1.5, were prepared by continuous NaCl gradient dialysis with urea. The latter method is also used in this report (method A). Partial confirmation for such conclusion is presented in Figure 2a from a complex with $r = 1.0$ in which there are only a slight reduction in the amplitude and no significant red shift in the peak and the crossover. However, for complexes with r of 2.0 or higher, there are a substantial red shift and a significant reduction of the 275-nm band similar to the CD changes observed for reconstituted nucleohistone H2B (Leffak et al., 1974) prepared by the same method of gradient dialysis, and for directly mixed polylysine-DNA (Chang et al., 1973) and polyarginine-DNA complexes (Yu et al., 1974). The substantial CD changes observed with complexes of $r = 2.0$ or higher could be related to the higher coverages of DNA by histone H4 in these complexes. According to the melting results shown in Figure 2a of Yu et al. (1976), at $r = 1.0$, only 15 to 20% of DNA base pairs are covered by histone H4, which could be too small a proportion of the total DNA to show substantial effect on DNA CD by histone H4 binding. Bigger CD effects become apparent only when more DNA base pairs are bound.

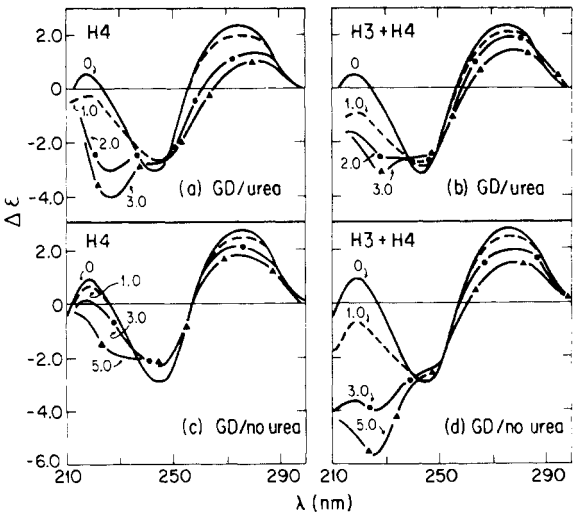

FIGURE 2: CD spectra of calf thymus histone H4-DNA and (H3 + H4)-DNA complexes prepared by NaCl gradient dialysis with urea (GD/urea) and without urea (GD/no urea). r value of each complex is included.

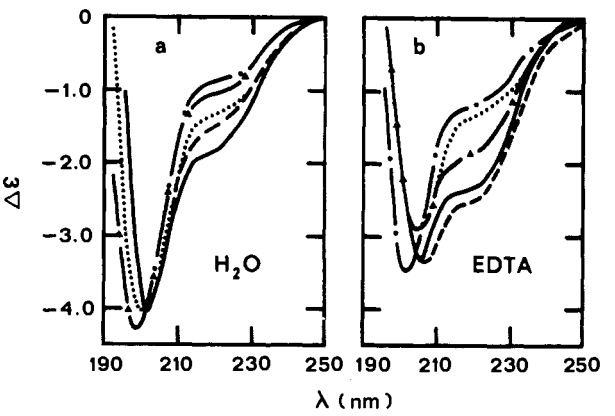

FIGURE 3: CD spectra of histone H3 fractions (a) in water and (b) in EDTA buffer. Calf thymus histone H3 (···); $(H3)_2$ (—); $(H3)_n$ (- - -); duck erythrocyte histone H3 (—●) and $(H3)_2$ (—▲).

It is noted that there is a substantial increase in light scattering of the complexes with higher r values, which could also make some contribution to the CD effects, as discussed more extensively by Shih and Fasman (1972).

The method of complex formation used by Shih and Fasman (1971) is different from the one used here, which could possibly contribute to the different CD properties shown in Figure 5 of Shih and Fasman (1971) and Figure 2a in this report, since CD properties are sensitive to both the salt concentrations at which urea is removed from the dialysis medium and the gradient of urea removal.

For histone (H3 + H4)-DNA complexes (Figure 2b), there are smaller changes in DNA CD (above 250 nm), both in amplitude and in red shift, than when histone H4 (Figure 2a) or histone H3 (Figure 1a) is used alone. In Figure 2b, the smaller red shift, both in the peak and in the crossover of this CD band, is similar to that in chromatin (Shih and Fasman, 1970), but the reduction in amplitude is much too small. The histone CD below 230 nm in the complexes made with the mixture seems to be closer to that of histone H4 complexes (Figure 2a) than to those of histone H3 (Figure 1a), as is true of the melting results discussed before (Yu et al., 1976).

CD results of histone H4-DNA and histone (H3 + H4)-DNA complexes prepared by gradient dialysis without urea (method B) are shown in Figure 2c and d, respectively. In histone H4-DNA complexes there are a smaller reduction in the DNA CD (above 250 nm) and a smaller contribution to the histone CD (below 230 nm) than are seen in the corresponding complexes prepared by gradient dialysis with urea (Figure 2a); this parallels the melting results (Yu et al., 1976), and could be explained as a result of aggregation of histone H4 in NaCl without urea. It is possible that nonspecific aggregation could prevent histone H4 from forming ordered secondary structures and thus hinder its binding to DNA. The presence of histone H3 in the mixture reduces the aggregation of the complexes (smaller light scattering), and at the same time causes a greater CD reduction above 250 nm and a more negative CD below 230 nm (Figure 2d).

Both melting (Yu et al., 1976) and CD results (Figures 1 and 2), therefore, suggest that, in NaCl media without urea, the presence of histone H3 could inhibit the nonspecific aggregation of histone H4 molecules and facilitate the formation of their individual secondary structures and subsequent interaction with DNA. On the other hand, in the presence of urea, the reverse seems to be true—that the presence of histone H4 improves the capacity of histone H3 to interact with DNA.

Complexes between DNA and Arginine-Rich Histones Prepared by Direct Mixing in EDTA Buffer. Figure 3 shows the CD spectra of histone H3 molecules in water (a) and in EDTA (b). In water, each spectrum has a major negative peak at about 200 nm, suggesting the presence of random coil structure. The shoulders near 220 nm in the CD spectra indicate some ordered structures in histone H3 in water since this shoulder disappears in the presence of 6.0 M urea or 6.0 M guanidine hydrochloride (Yu and Li, 1976). A small amount of ordered structures for histones in water or very low salt could possibly exist whenever there is a negative shoulder near 220 nm, for instance, in histone H1 (Fasman et al., 1970), histone H4 (Shih and Fasman, 1971; Li et al., 1971), histone H2A (IIb1 or f2a2) (D'Anna and Isenberg, 1974a), and H2B (D'Anna and Isenberg, 1974b).

Figure 3a also shows that, using the negative CD at 215 or 222 nm as the indicator of the content of ordered secondary structure, calf thymus histone H3 with two cysteine residues has more ordered structures than duck H3 with one. In each histone H3 species, the dimers with disulfide bond(s) have more ordered structures than the reduced histone H3 without this bond. This is not unreasonable because the formation of disulfide bond brings two molecules into close enough proximity that some intermolecular hydrogen bonds could be formed, such as those in parallel β-sheet. The smaller amplitude for the oligomer than for the dimer in water could result from some steric hindrance of hydrogen-bond formation or from the possible lack of full oxidation in all available-SH groups in the oligomers.

As is shown more clearly in Figure 5b, the addition of EDTA to histone H3 in water reduces the negative CD below 210 nm and increases that above. The difference CD spectrum shows a broad negative peak at around 215 nm. This difference spectrum suggests that the addition of EDTA buffer could induce the formation of more ordered secondary structures, primarily the β-sheet, in histone H3 (Holzworth and Doty, 1965; Greenfield and Fasman, 1969).

Figure 3b shows the CD spectra of various species of histone H3 in EDTA buffer before complex formation. As is the case in water, for both monomer and dimers, calf thymus histone H3 has more ordered structure than duck erythrocyte H3. The disulfide bond induces a more pronounced CD difference between the monomers and the dimers in EDTA than in water. This difference is also more pronounced in calf thymus than

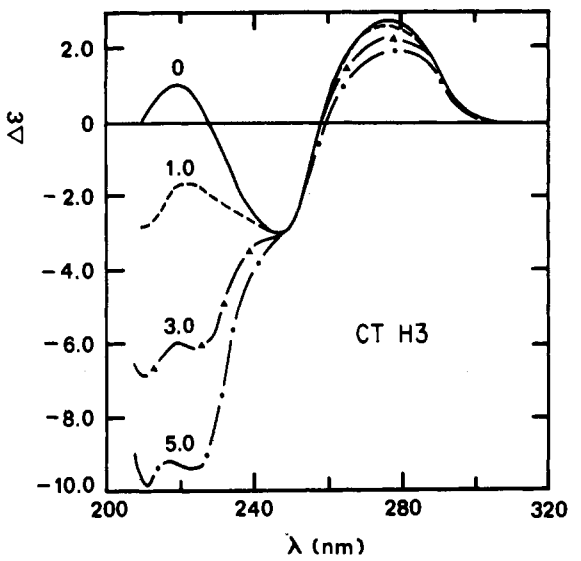

FIGURE 4: CD spectra of directly mixed calf thymus histone H3-DNA complexes in EDTA buffer. r value of each complex is included.

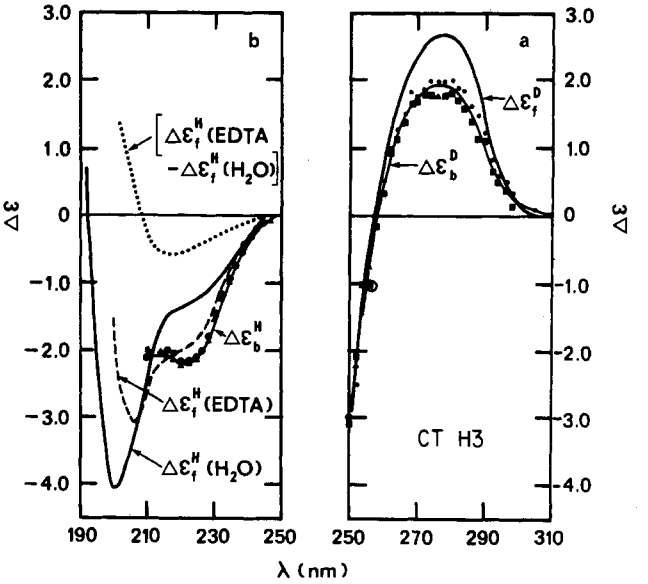

FIGURE 5: Calculated CD spectra of directly mixed calf thymus histone H3-DNA complexes. (a) $\Delta\epsilon_b^D$ of histone-H3-bound DNA base pairs calculated from eq 1 using complexes with r = 3.0 (●) and 5.0 (■). Also included is $\Delta\epsilon_f^D$ of pure DNA. $\Delta\epsilon$ is in M^{-1} cm^{-1} where M is moles/liter of nucleotide. (b) $\Delta\epsilon_f^H$ of free histone H3 in water (—), in EDTA buffer and dithiothreitol (- - -), and the difference of these two spectra (· · ·). $\Delta\epsilon_b^H$ of DNA-bound histone H3 calculated from eq 2 using complexes with r = 3.0 (●) and 5.0 (▲). $\Delta\epsilon$ is in M^{-1} cm^{-1} where M is moles/liter of amino acid residues of histones.

in duck erythrocyte. A consistent explanation of these results could be that the formation of disulfide bond facilitates the formation of intermolecular hydrogen bond by holding the two molecules close together. This formation of hydrogen bond is enhanced in EDTA buffer as compared with water, possibly due to the greater charge neutralization which could be affected by EDTA buffer.

Although calf thymus histone H3 oligomer in water has a smaller negative CD near 220 nm than the dimer, this order is reversed in EDTA buffer, indicating the presence of more secondary structure, probably β-sheet, in the oligomer than the dimer in EDTA buffer.

CD spectra of complexes between DNA and reduced calf thymus histone H3 are shown in Figure 4. The DNA CD band near 275 nm shows a reduction in amplitude and a very slight red shift. There are also big negative CD's near 220 nm corresponding to the bound histones. As far as the CD spectrum of native chromatin is concerned, complexes made by direct mixing in EDTA buffer are much closer to the native chromatin than are reconstituted complexes using NaCl gradient dialysis with or without urea.

Since both the melting (Yu et al., 1976) and CD results of these complexes are well defined, the CD spectra for both DNA base pairs bound by histone H3 ($\Delta\epsilon_b^D$) and those of bound histones ($\Delta\epsilon_b^H$) could be calculated, as done before for polylysine (Chang et al., 1973), protamine (Yu and Li, 1973), polyarginine (Yu et al., 1974), and poly(Lys^{40}, Ala^{60}) (Pinkston and Li, 1974). The following equations were used:

$$\Delta\epsilon = F\Delta\epsilon_b^D + (1 - F)\Delta\epsilon_f^D \quad (1)$$

$$\Delta\epsilon = \Delta\epsilon_f^D + r\Delta\epsilon_b^H \quad (2)$$

Equation 1 is valid for $\lambda > 250$ nm wherein the CD is contributed only by DNA, either free ($\Delta\epsilon_f^D$) or bound by histones ($\Delta\epsilon_b^D$). F in eq 1 is the fraction of base pairs bound by histones in each complex as determined from thermal denaturation (Yu et al., 1976). From eq 1, the CD of histone-bound base pairs, $\Delta\epsilon_b^D$, can be calculated.

Below 250 nm, eq 2 is used for calculating the CD of bound histone, $\Delta\epsilon_b^H$, by assuming that induced CD changes for DNA in this region are negligibly small compared with the CD contribution from histone.

Figure 5a shows the CD above 250 nm for free DNA, $\Delta\epsilon_f^D$, and for bound DNA, $\Delta\epsilon_b^D$, calculated from the measured results of two complexes of DNA with reduced calf thymus histone H3. Compared with $\Delta\epsilon_f^D$ of free DNA, $\Delta\epsilon_b^D$ is reduced by one-third in amplitude and is only slightly shifted to the red, judging from the crossover point. No shift of the peak is apparent.

The calculated CD spectrum of bound reduced histone H3 is given in Figure 5b which shows a negative peak near 220 nm. The change in amplitude of the CD near 220 nm from a free state, $\Delta\epsilon_f^H$ (EDTA), to a complexed state, $\Delta\epsilon_b^H$, is small for this histone.

The CD spectra of DNA complexed with various species of histone H3 are similar to those shown in Figure 4 and not presented here. Only the final spectra of bound histones ($\Delta\epsilon_b^H$) and DNA ($\Delta\epsilon_b^D$) bound by histones are shown respectively in Figures 6a and 6b. For bound histones, $\Delta\epsilon_b^H$, the results are similar to one another in shape but the amplitude varies slightly, following the order of calf thymus $(H3)_2$ > calf thymus $(H3)_n$ > calf thymus H3 > duck $(H3)_2$ > duck H3. The CD effect on DNA near 275 nm follows the same order except that the order for calf thymus $(H3)_n$ and $(H3)_2$ is reversed.

Previously, Wagner (1970) showed that, using the method of direct mixing in 0.001 M bicarbonate buffer (pH 7.2), the binding of histone H4 to DNA produces no change in the DNA CD near 275 nm. Similar observations were reported by Shih and Fasman (1971) if direct mixing were carried out in 0.01 and 0.14 M NaF. However, with direct mixing in EDTA buffer, as more histone H4 is complexed to DNA, the DNA CD near 275 nm is gradually reduced, but no red shift of this peak seems to be detectable, a result similar to that shown in Figure 4 for histone H3-DNA complexes. Since β = 5.2 amino acid residues/nucleotide for these complexes (Yu et al., 1976), a high r value is required to cover a substantial amount of DNA needed for induction of significant CD changes in DNA. The apparent contradictions may be attributable to the lower r values used in the previous experiments (Wagner, 1970; Shih

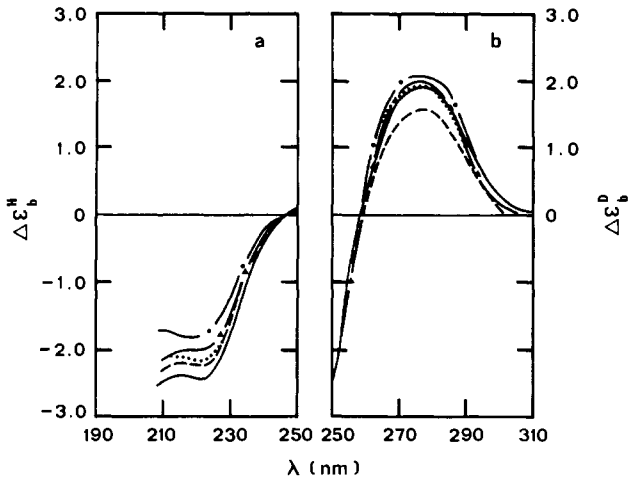

FIGURE 6: CD spectra of DNA-bound histone ($\Delta\epsilon_b^H$) and histone-bound DNA ($\Delta\epsilon_b^D$) calculated from directly mixed complexes in EDTA buffer. Calf thymus histone H3 (···), $(H3)_2$ (—), and $(H3)_n$ (- - -); duck erythrocyte histone H3 (—·—) and $(H3)_2$ (—▲).

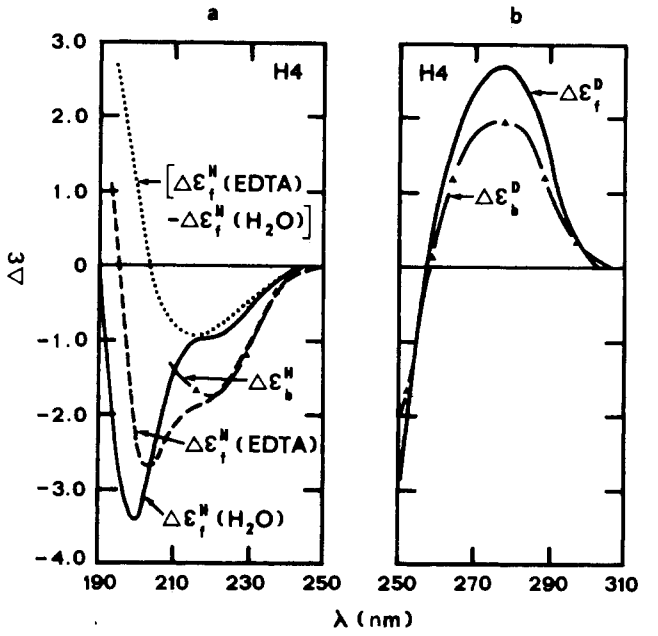

FIGURE 7: Calculated CD spectra of histone H4 and DNA in histone H4–DNA complexes prepared by direct mixing in EDTA buffer. (a) CD spectrum of free H4 in water (—), in EDTA (- - -), and their difference CD spectrum (···); CD spectrum of bound histone H4 ($\Delta\epsilon_b^H$) calculated from eq 2 (—▲). (b) CD spectrum of free DNA ($\Delta\epsilon_f^D$) (—) and DNA bound by histone H4 ($\Delta\epsilon_b^D$) calculated from eq 1 (—▲).

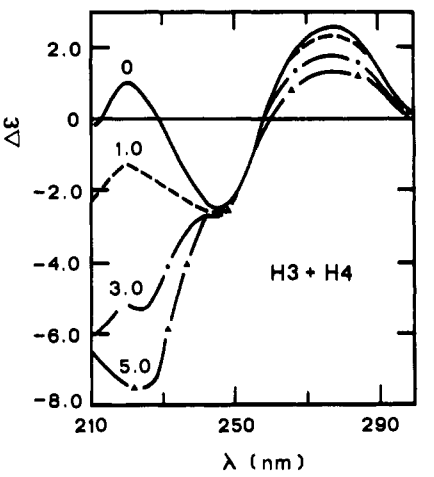

FIGURE 8: CD spectra of (H3 + H4)–DNA complexes in EDTA buffer prepared by method D. r value of each complex is included.

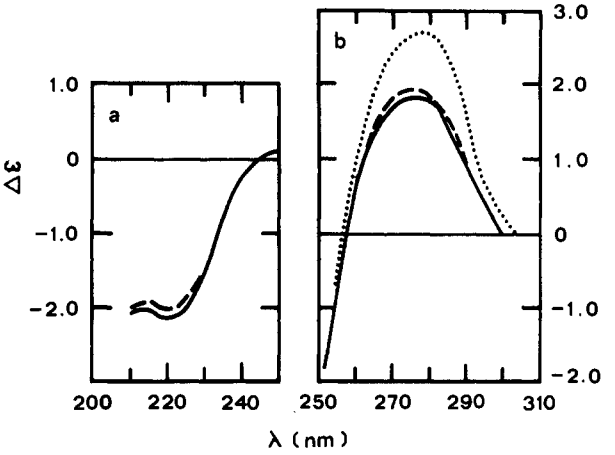

FIGURE 9: Calculated CD spectra of complexes of Figure 8. (a) Calculated CD spectra of bound histone H3 + H4 (—) and bound histone $(H3)_2$ + H4 (- - -) using eq 2; (b) calculated CD spectra of DNA bound by histone H3 + H4 (—) and histone $(H3)_2$ + H4 (- - -) using eq 1. Also included is the CD of free DNA (···).

and Fasman, 1971) as well as to the different histone conformations before complex formation.

The CD results of directly mixed histone (H3 + H4)–DNA complexes are similar to those of histone H4–DNA.

Using eq 1 and 2, the CD of bound histones H4 ($\Delta\epsilon_b^H$), and that of bound DNA ($\Delta\epsilon_b^D$) can be calculated. The results are shown in Figure 7. The calculated CD of bound histone H4 in EDTA buffer ($\Delta\epsilon_b^H$) coincides with the CD of free histone above 220 nm in the same buffer, $\Delta\epsilon_f^H$ (EDTA), but deviates below that wavelength. For the DNA CD near 275 nm, the calculated CD of bound DNA ($\Delta\epsilon_b^D$) is reduced in amplitude by 25% from that of pure DNA ($\Delta\epsilon_f^D$) with no detectable red shift, either in the peak or the crossover.

Complexes between DNA and Arginine-Rich Histones Prepared by Direct Mixing in Phosphate Buffer. Figure 8 shows CD results from complexes of DNA made with a mixture of H3 and H4 in phosphate buffer but dialyzed to EDTA buffer through the intermediate step of 0.1 M NaCl. The binding of these histones to DNA results in a reduction of the amplitude of the DNA CD near 275 nm with a slight red shift of the crossover and a substantial negative CD near 220 nm. Similar CD results were also obtained when a mixture of $(H3)_2$ and H4 was used. These CD spectra are similar to that of native chromatin (Shih and Fasman, 1970).

Using eq 1 and 2, both $\Delta\epsilon_b^H$ of the histones (below 250 nm) and $\Delta\epsilon_b^D$ of DNA bound by the histones (above 250 nm) can be calculated. The results are shown in Figures 9a and 9b, respectively. Binding of the histones to DNA causes a reduction of about 30% in the DNA CD band near 275 nm and only a very slight red shift of the crossover. The bound histones also show a negative CD band at 220 nm. Although quantitatively the effect of binding on the CD spectrum of both DNA and histone in these complexes is still smaller than the CD manifested by chromatin, qualitatively they are the same. Furthermore, of all the histone–DNA complexes described in reports to date, the complexes made between DNA and arginine-rich histones by direct mixing either in EDTA (Figures 4–7) or in phosphate buffer (Figure 8) provide the only conditions under which the CD properties of chromatin in both

DNA region (275-nm band) and histone region (220-nm band) can be reproduced to such an extent. In other reports, where various histones were complexed with DNA using different methods (Fasman et al., 1970; Wagner, 1970; Shih and Fasman, 1971; Li et al., 1971; Sponar and Fric, 1972; Adler et al., 1974, 1975; Leffak et al., 1974; Hwan et al., 1975), either the DNA CD or histone CD, or both of them, appears to be very different from the CD of native chromatin (Shih and Fasman, 1970; Simpson and Sober, 1970; Permogorov et al., 1970; Johnson et al., 1972; Chang and Li, 1974; Wilhelm et al., 1974; Hjelm and Huang, 1974; Li et al., 1975).

As mentioned earlier (Yu et al., 1976), the complexes made directly from DNA with a mixture of histones, H3 and H4 or $(H3)_2$ and H4 in EDTA buffer, show two melting bands of free and bound DNA with only one T_m' band at 83 °C for histone-bound DNA and have a β value of 6.0 amino acid residues/nucleotide. Presumably the less basic regions of histones are not directly bound to DNA under these conditions and therefore do not induce the intermediate melting band around 65 °C, as is the case when the H3 + H4 or $(H3)_2$ + H4 was preformed in phosphate buffer. Perhaps the reason for these differences can be understood from the study of the CD spectra of the unbound histones in water, EDTA, and phosphate buffer (Figure 10). The mixture of histones H3 and H4 in water shows a negative band at 200 nm and a shoulder near 220 nm. Solution in either EDTA or phosphate buffer induces substantial CD changes. Above 200 nm, the CD spectrum becomes more negative in phosphate than in EDTA buffer. If the difference CD spectrum is taken between the spectrum in water and that in EDTA or in water and that in phosphate buffer, one can compare the effects of these two buffers on histone conformations. The presence of EDTA buffer induces a broad difference CD spectrum with a peak around 215–220 nm, indicating the presence of both β-sheet and α-helix structures. On the other hand, the difference CD spectrum induced by phosphate buffer shows a major negative peak at 222 nm and a minor one at 210 nm, a spectrum very similar to that found for a transition from random coil to α-helix (Holzworth and Doty, 1965; Greenfield and Fasman, 1969; Li et al., 1972; Chen et al., 1972). Apparently, there is more α-helix in H3 + H4 placed in phosphate buffer than is formed by the same histones in EDTA buffer. It is also noted that, in 0.01 M phosphate buffer, there is substantial α-helical structure in histone H4 (Li et al., 1972) and in histone H3 (D'Anna and Isenberg, 1974c). The broad melting near 65 °C in DNA complexes made with histones H3 and H4 and the more pronounced band at this temperature in those complexes made with a mixture of H3 and H4 or $(H3)_2$ and H4 (Yu et al., 1976) indicate the importance of α-helical structure for the less basic regions of these histones to bind DNA correctly. The presence of β-sheet structure in histone H3 or H4 or their mixtures in EDTA buffer, perhaps, prevents the less basic regions of these histones from a proper binding to DNA, because no such intermediate melting band has been detected in DNA complexes made with these histones in EDTA buffer. These conclusions are in accord with the previous observation that, in native chromatin, most of the α-helical structure in histones was found in the less basic regions of histones (Li et al., 1975).

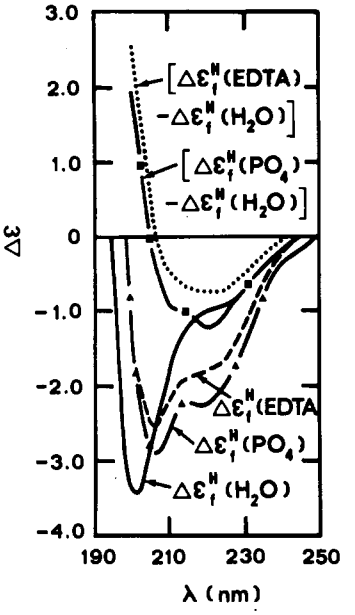

FIGURE 10: Circular dichroism of free histone H3 + H4 in various conditions. In water (—), in EDTA buffer (- - -), and its difference CD spectrum (· · ·); in 0.01 M phosphate buffer (—▲) and its difference CD spectrum (—■).

Discussion

Recent reports on chromatin structure studied by electron microscopy (Olins and Olins, 1974; Griffith, 1975; Oudet et al., 1975), nuclease digestion (Clark and Felsenfeld, 1971; Rill and Van Holde, 1973; Noll, 1974; Sollner-Webb and Felsenfeld, 1975; Axel, 1975), and neutron diffraction (Baldwin et al., 1975) emphasize the gross structures of histone-bound fragments in chromatin. Complementary to these reports on chromatin structure are thermal denaturation and circular dichroism studies of chromatin. Thermal denaturation measures the thermal stability of DNA bound by histones which is very sensitive to the direct binding of histones to DNA, in particular the direct interaction between basic amino acid residues in histones and phosphates in DNA. Circular dichroism, on the other hand, measures the conformations of both bound histones and bound DNA in chromatin. Thus both thermal denaturation and CD provide information of histone–DNA interaction in chromatin beyond what can be revealed by other techniques. Without considering these results would inevitably make the picture of chromatin structure less clear than it should be. For instance, after incorporating both thermal denaturation and CD results of chromatin with those obtained from electron microscopy, nuclease digestion, neutron diffraction, and electrophoresis, a model of chromatin structure was proposed (Li, 1975) which can explain more observable phenomena of chromatin than can the other models (Kornberg, 1974; Van Holde et al., 1974; Baldwin et al., 1975).

As shown in Figures 1, 2, 4, and 8 in this report and Figures 1, 2, 3, and 6 in the earlier one (Yu et al., 1976), both thermal denaturation and CD properties of a histone–DNA complex depend greatly upon how this complex is prepared. In other words, the physical properties of a histone–DNA complex are influenced greatly by the conditions and, in turn, the secondary, tertiary, and quaternary structures of histones and the secondary structure of DNA before complex formation. Both thermal denaturation and CD results of arginine-rich histone–DNA complexes in these two reports indicate that those complexes showing better melting properties also show CD properties closer to those of native chromatin (both histone CD near 220 nm and DNA CD near 275 nm). Since melting properties measure the efficiency of coverage on DNA by histones and the quality of binding to DNA by both the more basic and the less basic regions of histones, the results in these two reports strongly suggest that the formation of proper

secondary structure (more likely to be α-helix in the less basic regions of histones) is extremely important for a proper binding of histones to DNA. It could also be crucial for the formation of correct histone subunits—either in free state or in bound state in chromatin.

The CD spectra shown in Figures 4–9 in this report indicate that arginine-rich histones, H3 and H4, play an equally important role in maintaining conformation and structure of chromatin which is in agreement with the earlier conclusion that the CD properties of chromatin are mainly contributed by arginine-rich histones (Simpson and Sober, 1970; Hanlon et al., 1974) or by arginine-rich and slightly lysine-rich histones (Leffak et al., 1974; Hjelm and Huang, 1974; Li et al., 1975).

The variation of both DNA and histone CD in histone H3–DNA complexes using reduced or oxidized histone H3 either from calf thymus or from duck erythrocytes (Figures 3, 6, and 7) indicates the sensitivity of the conformation of the complexes upon the formation of disulfide bond and the presence of one or two cysteine residues in histone H3. Compared with thermal denaturation results of these complexes (Figure 3 and Table I in Yu et al., 1976), CD is more sensitive in probing the differences of complex properties resulting from minor variation in histone.

It was reported by Kornberg and Thomas (1974) that it was important in preparation procedures to avoid denaturation of histones by the use of acids. This seems to be unnecessary, because it has been a well-known fact that histones, extracted by acid and purified under denaturation conditions, can be used for reconstitution with DNA and the resulting properties of these complexes are very similar to those in chromatin (Bekhor et al., 1969; Huang and Huang, 1969; Ansevin and Brown, 1971; Li and Bonner, 1971; Stein et al., 1975). In this report and the earlier one (Yu et al., 1976), it was also shown that substantial melting properties and secondary structures of both histones and DNA in chromatin can be reproduced, using purified histones which have been denatured during purification. In native and partially dehistonized chromatin, it is also possible to restore the melting and conformational properties after denaturation of histone structures by 5.0 M urea (Chang and Li, 1974). Histone subunits (D'Anna and Isenberg, 1975d) and cross-linkage among histones (Martinson and McCarthy, 1975), forming structures similar to those observed in chromatin, can also be reproduced by using predenatured histones.

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Modification of Histone Binding in Calf Thymus Chromatin and in the Chromatin–Protamine Complex by Acetic Anhydride†

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ABSTRACT: A relationship between side-chain modification of histones and their displaceability from DNA has been investigated using calf thymus chromatin which was chemically acetylated with acetic anhydride. When the chromatin is treated with increasingly higher concentrations of the reagent, histones become acetylated to an increasingly greater extent, attaining the modification at 23–24 sites for histone I, 5–6 for IIb_1, 9–10 for IIb_2, 5–6 for III, and 3–4 for IV. As the chromatin becomes more acetylated, NaCl concentrations required for histone removal are lowered. Saturation binding of protamine does not bring about either an increase in the number of acetylation sites of histones in chromatin or a decrease of the NaCl requirement for dissociation of the acetylated chromatins. A comparison of the present results with the extents of histone acetylation known to occur enzymatically in vivo indicates that the complete removal of somatic histones during transformation of chromatin in spermiogenesis cannot be explained on the basis of decreased binding of the histone to DNA by acetylation or by a combination of acetylation and protamine binding, suggesting that the displacement process may require some additional processes.

The process of histone displacement during the transformation of nucleohistone into nucleoprotamine in spermiogenesis may involve a combination of different mechanisms which would weaken interactions between histones and DNA. When nucleohistone is exposed in vitro to protamine, the protein binds stoichiometrically to DNA, weakening the electrostatic interactions between histones and DNA (Wong and Marushige, 1975). The saturated binding of protamine does not, however, cause major displacement of any of the histone fractions (Wong and Marushige, 1975), and only histone I is completely dissociated from the DNA by addition of excess protamine (Evans et al., 1970; Marushige and Dixon, 1971; Wong and Marushige, 1975). Phosphorylation (Marushige et al., 1969; Sung and Dixon, 1970; Louie and Dixon, 1972) and acetylation (Candido and Dixon, 1971, 1972a–c) of histones have been observed in developing trout testes. These modifications which are found in the spermatids (Marushige et al., 1969; Candido and Dixon, 1972b) are possibly associated with the replacement process. In an attempt to elucidate the mechanisms involved in the displacement of the histones during the replacement process, a further investigation of the chemical acetylation of chromatin, first described by Simpson (1971), has been done. The relationship between the extent of side-chain modification of histones and their displaceability from DNA is examined in conjunction with protamine binding to chromatin.

Materials and Methods

Preparation of Chromatin. Calf thymus chromatin was prepared by the method of Marushige and Bonner (1966) with a modification previously described (Wong and Marushige, 1975), and stored in 50% glycerol at −20 °C. Just before use, the chromatin was diluted five times with 5 mM sodium borate (pH 8.2) and centrifuged at 17 000*g* for 20 min. The sediment was suspended in the borate buffer at a concentration equivalent to approximately 1 mg of DNA/ml and then sheared in a Waring blender at 100 V for 3 min. After centrifugation at 17 000*g* for 20 min, the supernatant was removed and used as the chromatin.

Calf thymus chromatin–protamine complex was prepared by dropwise addition of concentrated protamine solution to the calf thymus chromatin solution which was vigorously stirred in an ice bath. The final concentration of protamine was ad-

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