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Interactions between Arginine-Rich Histones and Deoxyribonucleic Acids. I. Thermal Denaturation†

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ABSTRACT: Physical properties of histone–DNA complexes very often depend upon the method of complex formation. In an attempt to make the studies of histone–DNA interactions more relevant to biological systems, results from thermal denaturation of native chromatin were used as references for determining how closely a given histone–DNA complex approaches its native state in chromatin. In the case of arginine-rich histones H3 (III or f3) and H4 (IV or f2a1), four methods were used for making complexes with calf thymus DNA: (A) NaCl gradient dialysis with urea; (B) NaCl gradient dialysis without urea; (C) direct mixing in 2.5×10^{-4} EDTA, pH 8.0; and (D) direct mixing in 0.01 M sodium phosphate, pH 7.0. It was observed that a complex made by direct mixing in phosphate (method D) is closer to the native than is one made by direct mixing in EDTA (method C) than the one made by gradient dialysis with urea (method A) or without urea (method B). Regardless of the method used for complex formation, no substantial differences were observed between complexes with histone H3 dimer with disulfide bond(s) and a reduced H3 without disulfide bond, implying that perhaps a dimer with or without disulfide bond is a natural fundamental subunit in our experimental conditions. When the method of direct mixing in EDTA is used, the melting properties of the complexes vary only slightly with any one of the following H3 histones: from calf thymus, H3 without disulfide bond, H3 dimer, and H3 oligomer with disulfide bonds, also, from duck erythrocyte, H3 monomer and dimer. The complexes formed between DNA and a mixture of H3 and H4 by method D have melting properties similar to those of native chromatin. Since an equimolar mixture of histone H3 and H4 in 0.01 M phosphate, pH 7.0, was shown to form a tetramer (D'Anna, J. A., and Isenberg, I. (1974), *Biochem. Biophys. Res. Commun. 61,* 343), our results suggest that, a tetramer of H3 and H4, likely to be $(H3)_2(H4)_2$, formed from one H3 dimer and one H4 dimer, can bind DNA in a manner similar to that in native chromatin.

Histone binding to DNA in chromatin induces two phases of melting at higher temperatures (Li and Bonner, 1971; Ansevin et al., 1971; Li et al., 1973; Tsai et al., 1975). This phenomenon has been interpreted as a result of the binding to DNA of both the less basic and the more basic regions of histones (Li and Bonner, 1971). Such binding also causes a reduction of the positive circular dichroism (CD) band near 275 nm for DNA and generated a large negative CD band near 220 nm for bound histones (Shih and Fasman, 1970; Simpson and Sober, 1970; Permogorov et al., 1970; Johnson et al., 1972; Chang and Li, 1974; Wilhelm et al., 1974). Of the individual histones, histone H1 (I or f1) seems to make the least contribution to the spectrum both with respect to reduction of DNA CD near 275 nm (Simpson and Sober, 1970; Hjelm and Huang, 1974; Hanlon et al., 1974; Li et al., 1975) and the CD of bound histone near 220 nm (Li et al., 1975). The less basic (hydrophobic) regions of histones seem to have more contribution to the negative CD near 220 nm, and consequently more ordered secondary structures, than do the more basic regions (Li et al., 1975). Both the thermal denaturation and the CD properties of chromatin are well characterized, and together they can be used respectively as criteria for assessing the resemblance of reconstituted chromatin or a histone–DNA complex to the original native chromatin.

Studies on histone–DNA interaction using purified histones and DNA have been reported for histone H1 (I or f1) (Olins, 1969; Fasman et al., 1970; Sponar and Fric, 1972), on histone H4 (IV or f2a1) (Wagner, 1970; Shih and Fasman, 1971; Li et al., 1971; Adler et al., 1975), histone H2B (IIb2 or f2b) (Adler et al., 1975; Leffak et al., 1974), and histone H5 (V or f2c) (Hwan et al., 1975; Tsai et al., 1975). Interaction between histone H3 (III or f3) and DNA has not been studied extensively before. Furthermore, it is well known that different methods of preparing histone–DNA complexes are likely to produce different physical properties even if the same histone was used. Nevertheless, no serious attempt has been made to

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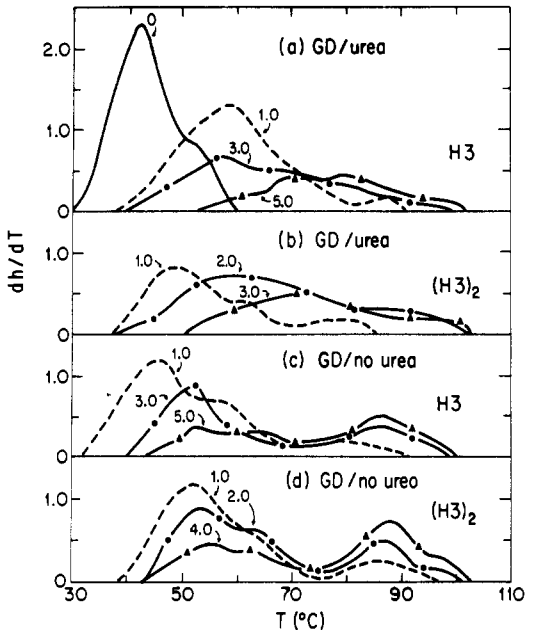

FIGURE 1: Derivative melting profiles of calf thymus histone H3-DNA and $(H3)_2$-DNA complexes prepared by NaCl gradient dialysis with urea (GD/urea) or without urea (GD/no urea). r value of each complex is indicated.

examine these differences.

In this communication, we report thermal denaturation results obtained from histone H3-DNA, H4-DNA, and (H3 + H4)-DNA complexes made either by NaCl gradient dialysis with or without urea, or by direct mixing at low ionic strength. The properties of these types of complex were compared with those obtained from native chromatin. Histone H4 from calf thymus, H3 monomer and dimer from calf thymus and duck erythrocytes, and oligomers from calf thymus were used. Since the histone H3 dimer containing one or two disulfide bonds must occur in the C-terminal region (positions 96 and 110 in calf thymus histone H3) (DeLange et al., 1973; Hnilica, 1972), the two monomers in the dimer are expected to be in a parallel configuration. As to be shown in this report and the following one (Yu et al., 1976), complexes made from either histone H3 without disulfide bond or with disulfide bonds are essentially the same, which suggests that histone H3 dimers with or without disulfide bonds are the most stable subunits in solution and possibly in chromatin also.

Materials and Methods

Purification of calf thymus histone H3 monomer, dimer, and oligomers and duck erythrocyte histone H3 monomer and dimer was carried out as described elsewhere (Yu and Li, 1976). Histone H4 was purified as described by Shih and Fasman (1971).

Four different methods of complex formation were used. Method A was gradient dialysis with urea: continuous NaCl gradient dialysis (2.0 to about 0.1 M) in 5 M urea, followed by a continuous urea gradient dialysis (5.0 to 0 M) (Li and Bonner, 1971), a modification of the stepwise gradient dialysis first used by Bekhor et al. (1969) and by Huang and Huang (1969). Method B was gradient dialysis without urea: a similar NaCl gradient dialysis (2.0 to about 0.1 M) without urea, a modification of the stepwise gradient dialysis first used by Huang et al. (1964). In both methods, the final complexes were dialyzed to 2.5×10^{-4} EDTA, pH 8.0 (EDTA buffer), for measurements. Method C used direct mixing in 2.5×10^{-4} M EDTA, pH 8.0, a slow dropwise addition of histone H3 to DNA in this buffer. In method D, equimolar quantities of histone H4 and H3 (with or without disulfide bond) were mixed in water. Appropriate amounts of phosphate buffer (0.1 M sodium phosphate, pH 7.0) were added to a final phosphate concentration of 0.01 M. The solution was left in ice for at least 1 h before complexing with DNA, also buffered in 0.01 M phosphate. After complexing, portions of the complexes were first dialyzed overnight against 0.1 M NaCl, 0.01 M Tris, pH 8.0, and then overnight against 2.5×10^{-4} M EDTA buffer, pH 8.0 (EDTA), except for those remaining in phosphate buffer. Use of the intermediate step of dialysis against 0.1 M NaCl with Tris somehow facilitates the removal of sodium phosphate from the medium. To proceed from 0.01 M phosphate directly to EDTA buffer seemed to require a prolonged dialysis of about 1 week to lower the melting temperature of free DNA (73 °C in 0.01 M phosphate buffer) to that normally observed in EDTA buffer (about 47 °C).

The concentration of histones in water was determined by measuring the absorbance at 230 nm using $A_{230} = 4.25$ for 1 mg/ml histone solution (Ohlenbusch et al., 1967), further confirmed by Lowry's method (Lowry et al., 1951), and by the amino acid analyzer. After the concentration had been determined in water, the histone solution was made into either 2.0 M NaCl with 5.0 M urea, 2.0 M NaCl, 2.5×10^{-4} M EDTA, or 0.01 M phosphate, depending upon the method of complex formation used.

Calf thymus DNA purchased from Sigma Chemical Co. was purified by phenol extraction. A molar extinction coefficient of 6500 M^{-1} cm^{-1} in nucleotide at 260 nm was used for both DNA and complexes. The input ratio (r) of histone to DNA is reported in amino acid residues per nucleotide. Whenever reduced histone H3 was used, a reducing agent of 5×10^{-3} M dithiothreitol was added in the media to prevent oxidation.

Thermal denaturation of the complexes was made on a Gilford Spectrophotometer Model 2400-S. Percent increase in absorbance at 260 nm is reported as hyperchromicity (h) and its first derivative with temperature as dh/dT.

Results

Complexes of DNA and Arginine-Rich Histones Prepared by NaCl Gradient Dialysis. Thermal denaturation results from reduced histone H3-DNA complexes prepared by NaCl gradient dialysis with urea are shown in Figure 1a and that from H3 dimer-DNA in Figure 1b. Except for a generalized broad melting at higher temperature with complexes of greater r value, there is no definitive melting band which can be assigned to base pairs bound by histone H3. Although two or three bands could be roughly distinguished for each complex, the peak positions of these bands shift greatly from one complex to another. Peak positions of these bands from different preparations also vary. These results are different from those obtained from chromatin (Li and Bonner, 1971; Ansevin et al., 1971), directly mixed histone-DNA complexes in 3.6 M urea (Ansevin and Brown, 1971; Tsai et al., 1975), or from reconstituted complexes of DNA with histone H1 (Shih and Bonner, 1970), histone H2A (IIb1 or f2a2) + H2B (IIb2 or f2b) (Li and Bonner, 1971), histone H2B (Leffak et al., 1974), or histone H5 (V or f2c) (Hwan et al., 1975), in which definitive melting bands corresponding to histone-bound and histone-free regions can be assigned, even though similar gradient dialysis methods were used for all the reconstituted complexes.

The similar results in Figures 1a and 1b indicate that, qualitatively, both reduced histone H3 and histone H3 dimer

yield complexes which are similar as far as melting properties are concerned.

Figure 2a shows this derivative melting profiles of histone H4–DNA complexes of varied input ratios of histone to DNA, and Figure 2b shows those of histone (H3 + H4)–DNA complexes also prepared by NaCl gradient dialysis with urea. For histone H4–DNA complexes, the results are similar to those reported earlier (Shih and Bonner, 1970) in which the binding of histone to DNA produces a new melting band at about 85 °C. Although the profiles are still different from those of chromatin, since they lack intermediate band at about 65–75 °C observed in chromatin under the same melting conditions (Li and Bonner, 1971; Li et al., 1973), the distinction between the melting bands of free as well as histone-bound DNA is better than that in histone H3–DNA complexes prepared by the same procedure (Figures 1a and 1b).

The complexes made with a mixture of histones H3 and H4 show melting properties similar to those of histone H4–DNA complexes but quite different from those of histone H3–DNA complexes (Figure 1a). These results suggest that, although the presence of histone H3 does not seem to improve the binding of histone H4 to DNA, the presence of histone H4 does reduce the poor melting properties observed when histone H3 is used along.

The rationale for using NaCl gradient dialysis with urea (Bekhor et al., 1969; Huang and Huang, 1969) is that urea is expected to reduce histone–histone interaction as demonstrated in the case of histone H4 (Li and Isenberg, 1972). Urea can reduce not only hydrophobic interaction but also hydrogen-bond formation in both α-helix and β-sheet structures. If a certain native histone in the nucleus requires specific secondary, tertiary, or quaternary structures in order to bind DNA in a characteristic manner, the use of urea might destroy such specific interaction. This may be responsible for the great difference in the melting (Figure 1) and CD properties (Yu et al., 1976) of arginine-rich histone–DNA complexes made by NaCl gradient dialysis with urea and those obtained from chromatin. In order to see whether the removal of urea from the media changes these properties, continuous NaCl gradient dialysis without urea was used for complexing.

Figures 1c and 1d respectively show melting results of complexes with both reduced histone H3 and histone H3 dimer. Although the two melting bands induced by histone-binding in chromatin (Li and Bonner, 1971; Ansevin et al., 1971; Li et al., 1973; Tsai et al., 1975) are not reproduced, at least a definitive separation of melting of the histone-free (45–60 °C) and histone-bound regions (80–100 °C) was achieved. Apparently, as far as histone H3 is concerned, the use of urea in the medium does not improve conditions for complex formation. Again, with respect to melting properties, the results in Figures 1c and 1d show that both reduced histone H3 and oxidized H3 dimer yield similar complexes with DNA.

Histone H4–DNA complexes formed by gradient dialysis without urea show visible precipitates. Thermal denaturation results (Figure 2c) show that only very small proportions of the DNA base pairs are really covered by histone H4, even when the r of the complex is as high as 5.0. This suggests that most of the histone H4 molecules are able to engage in mutual interaction or aggregation but not with DNA. In fact, it is well known that histone H4 can form large aggregates in salt (Edwards and Shooter, 1969; Boublik et al., 1970; Pekary et al., 1975a). The presence of histone H3 seems to reduce the tendency for aggregation and enhance the melting of histone-bound regions at higher temperatures (Figure 2d). A comparison of the melting results of histone H4–DNA complexes in Figures 2a and 2c shows that the use of urea in NaCl gradient dialysis tends to reduce histone H4 aggregation and increase its binding to DNA. In fact, it has been shown that urea does indeed reduce the formation of rigid structures of histone H4 in NaCl (Li and Isenberg, 1972).

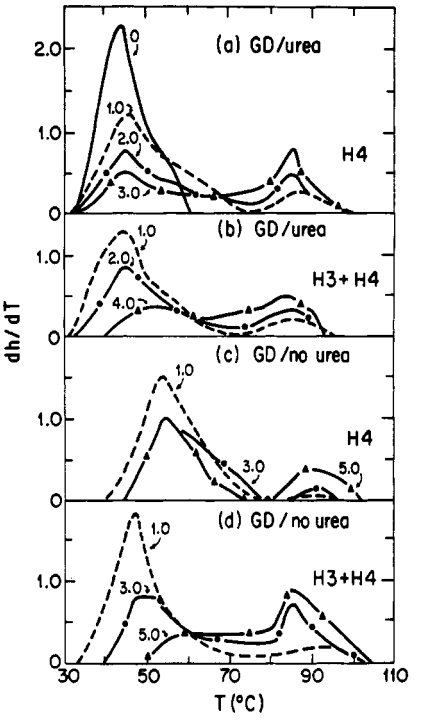

FIGURE 2: Derivative melting profiles of calf thymus histone H4–DNA and (H3 + H4)–DNA complexes prepared by NaCl gradient dialysis with urea (GD/urea) or without urea (GD/no urea). r value of each complex is included.

Complexes between DNA and Arginine-Rich Histones Prepared by Direct Mixing in EDTA Buffer. Melting results of direct-mixed complexes between DNA and reduced histone H3, oxidized H3 dimer, or oligomer from calf thymus and between DNA and histone H3 dimer from duck erythrocytes are shown in Figure 3. Though not shown here, the results of complexes with reduced histone H3 from duck are similar to those with duck histone H3 dimer. Biphasic melting curves are very clear in all these cases with a melting band near 50 °C (T_m) corresponding to free base pairs, and another band near 90 °C (T_m') corresponding to histone-bound base pairs. A complex with higher r value shows a greater amplitude in the melting band at T_m' with a simultaneous decrease of the melting band at T_m. Except for minor variations, the melting curves of these complexes using various histone H3 are similar to one another. The slightly lower melting temperatures for both the T_m and the T_m' in the complexes with reduced histone H3 of calf thymus could be due to the presence of reducing agent dithiothreitol in the melting medium.

The melting results of histone H4–DNA complexes prepared by direct mixing in EDTA buffer are similar to those of histone H3–DNA complexes. The binding of histone H4 to DNA induces a definitive melting band at 87 °C (T_m'), about 3 °C lower than that in H3–DNA complexes. There is no intermediate band between 65 and 75 °C such as is observed in chromatin in the same melting medium (Li and Bonner, 1971; Li et al., 1973). The presence of histone H3 in the mixture of H3 + H4 yields similar melting results except for a slight decrease (2–4 °C) in the melting temperature of histone-bound regions.

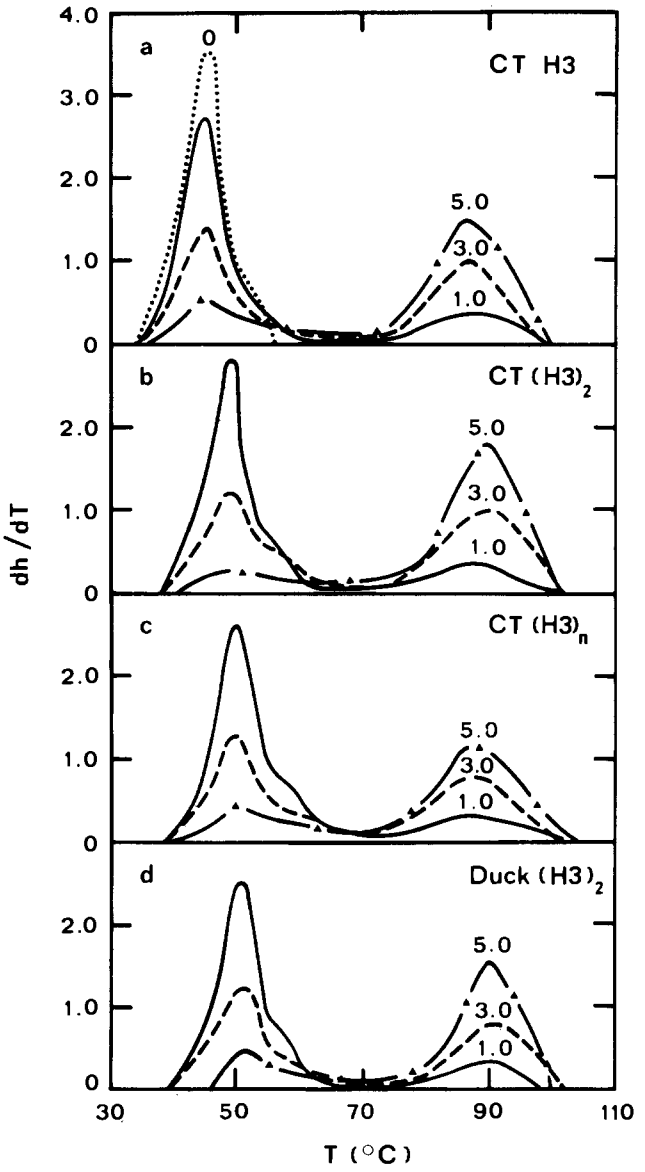

FIGURE 3: Derivative melting profiles of histone H3-DNA complexes prepared by direct mixing in EDTA buffer. Histone fraction used and r value of each complex are indicated.

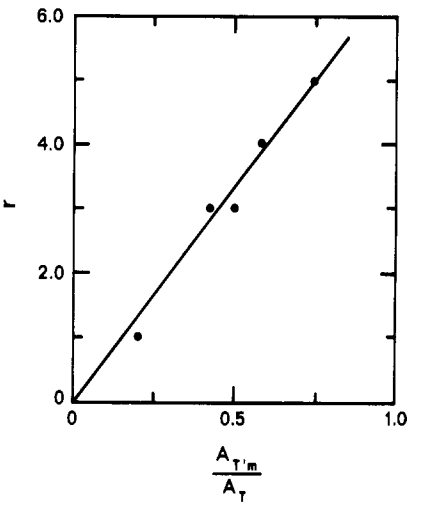

FIGURE 4: Linear plot of eq 2 using directly mixed calf thymus histone H3-DNA complexes.

Table I: The β Values of Various Arginine-Rich Histone-DNA Complexes Prepared by the Method of Direct Mixing.

Method	Complexes	β (Amino Acids / Nucleotide)
Direct mixing in EDTA	Calf thymus H3	6.5
	Calf thymus $(H3)_2$	5.6
	Calf thymus $(H3)_n$	5.8
	Duck erythrocyte H3	6.4
	Duck erythrocyte $(H3)_2$	5.9
	Calf thymus H4	5.2
	Calf thymus H3 + H4	6.0
Direct mixing in 0.1 M phosphate[a]	Calf thymus H3	4.0
	Calf thymus H4	4.0
	Calf thymus H3 + H4	3.1
	Calf thymus $(H3)_2$ + H4	3.1

[a] The complexes were dialyzed to 0.1 M NaCl, 0.01 M Tris, pH 8.0, and finally to 2.5×10^{-4} M EDTA, pH 8.0.

Since the melting curves are well separated and consistent among complexes with varied r values, it is possible to use the following equations to calculate the fraction of base pairs bound by histone (F) and the number of amino acid residues of histone per nucleotide of DNA in histone-bound regions (β) in these complexes (Li, 1973a).

$$F = A_{T_m'}/A_T \quad (1)$$

$$r = \beta F = \beta(A_{T_m'}/A_T) \quad (2)$$

where $A_{T_m'}$ and A_T are respectively the melting areas under the T_m' band and under the whole melting curve.

The linear plot of eq 2 for the complexes of DNA with reduced histone H3 from calf thymus is shown in Figure 4. The slope (β) is determined to be 6.5 amino acid residues per nucleotide. The β values of the various complexes are given in Table I and vary only slightly among histone H3 complexes. That of the reduced histone H3 is about 10–20% higher than that of the dimer whether calf thymus or duck erythrocyte H3 is used. There is no significant difference in β values between calf thymus and duck erythrocyte H3, although 80% of calf thymus H3 molecules have two -SH groups per molecule, and the other 20% have one (Patthy et al., 1975), whereas all duck erythrocyte H3 molecules have only one (Brandt et al., 1974).

The β value is 5.2 amino acid residues per nucleotide for histone H4-DNA complexes and 6.0 for those of histone (H3 + H4)-DNA (Table I). The latter is between 5.2 for histone H4-DNA and 6.5 for histone H3-DNA prepared by the same method (Table I). This implies that, in EDTA buffer, histones H3 and H4 could interact with DNA independently.

Complexes between DNA and Histones H3, H4, (H3 + H4), or [(H3)$_2$ + H4] Prepared by Direct Mixing in Phosphate Buffer. At low concentration of phosphate buffer, histone H4 forms some α-helices and β-sheets (Li et al., 1972). Based upon the consideration of kinetics and the amino acid sequence of H4, a parallel dimer with β-structure is favored over antiparallel configuration (Li, 1973b). Formation of α-helix and β-sheet has also been observed for histone H3 (D'Anna and Isenberg, 1974a). In addition, an equimolar mixture of histone H3 and H4 in phosphate buffer forms tetramers (D'Anna and Isenberg, 1974c). If a tetramer of H3 and H4 is a subunit in chromatin, as has been suggested (Kornberg, 1974; Li, 1975), it could be possible to produce some of the

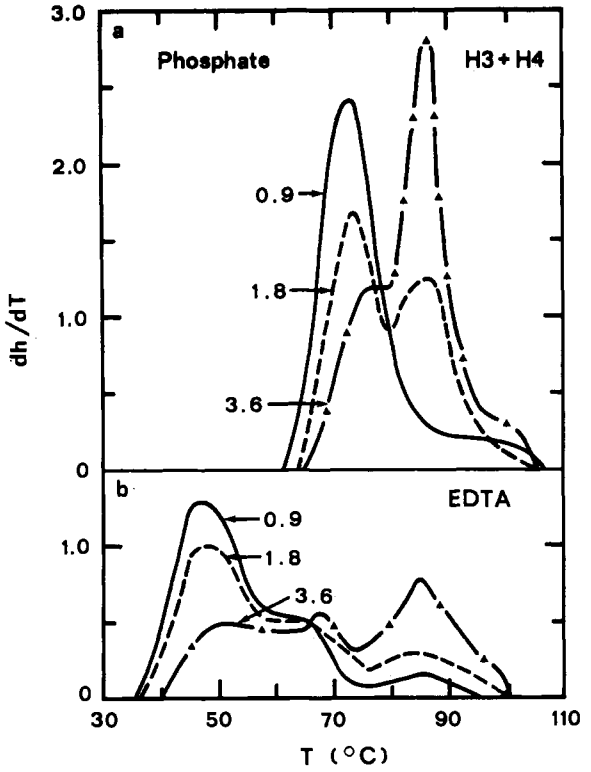

FIGURE 5: Derivative melting profiles of calf thymus histone (H3 + H4)-DNA complexes prepared by direct mixing in 0.01 M phosphate buffer. (a) Melting medium is 0.01 M phosphate, pH 7.0; (b) complexes of a, but dialyzed directly into EDTA buffer for about a week. *r* value of each complex is indicated.

physical properties of chromatin, such as thermal denaturation and CD, from complexes made between this tetramer and DNA. Furthermore, in order to see whether a histone H3 dimer is a more fundamental subunit of the tetramer as suggested (Li, 1975), histone H3, with or without disulfide bond(s) (i.e., monomer or dimer according to electrophoretic criterion), was used. It was considered that if they should yield the same results, then histone H3 dimer would be implicated as a basic subunit. Finally, in order to better understand the possible arrangement of components in the tetramer, complexes between DNA and individual histones, H3 or H4, were also examined.

Figure 5a shows thermal denaturation results of directly mixed complexes between DNA and an equimolar mixture of histones H3 and H4 in 0.01 M phosphate, pH 7.0. Each melting curve can be divided approximately into two regions, one at 73 °C, representing free DNA, and another at 87 °C with a shoulder at about 100 °C. Since the well-defined melting bands of chromatin in EDTA buffer lose their resolution at higher ionic strength (0.01 M Na^+, for example) (Li and Bonner, 1971), it is difficult to compare the melting properties of these complexes in phosphate buffer with the native chromatin in EDTA. Therefore, after making the complexes in 0.01 M phosphate buffer, they were dialyzed immediately into EDTA buffer. To our surprise, the lower melting band at 73 °C in phosphate buffer could not easily be lowered to 47 °C, the melting temperature of free DNA in EDTA buffer. Just why this should be so is unclear because a long dialysis was also found to be necessary to lower the melting temperature of uncomplexed DNA. Figure 5b shows the final results after 1 week's dialysis with many changes of EDTA buffer. It can be seen that, in addition to the melting bands near 47 and 85 °C in EDTA buffer, there is substantial melting around 60–70 °C, which is similar to melting band III in chromatin (66 °C in pea bud and 73 °C in calf thymus), a melting band assigned to DNA bound by the less basic regions of histones (Li and Bonner, 1971; Li et al., 1973).

Since 1 week's dialysis seemed too long to be practical, the complexes made in phosphate buffer were first dialyzed overnight against 0.1 M NaCl, 0.01 M Tris, pH 8.0, and then against EDTA buffer. Sodium chloride was introduced with the intention of forcing out sodium phosphate from the DNA or from the medium inside the dialysis bag. Figure 6 shows melting results of complexes prepared by this procedure, using histone H4 alone (a), H3 alone (b), an equimolar mixture of H3 and H4 (c), and of $(H3)_2$ and H4 (d). It is emphasized that,

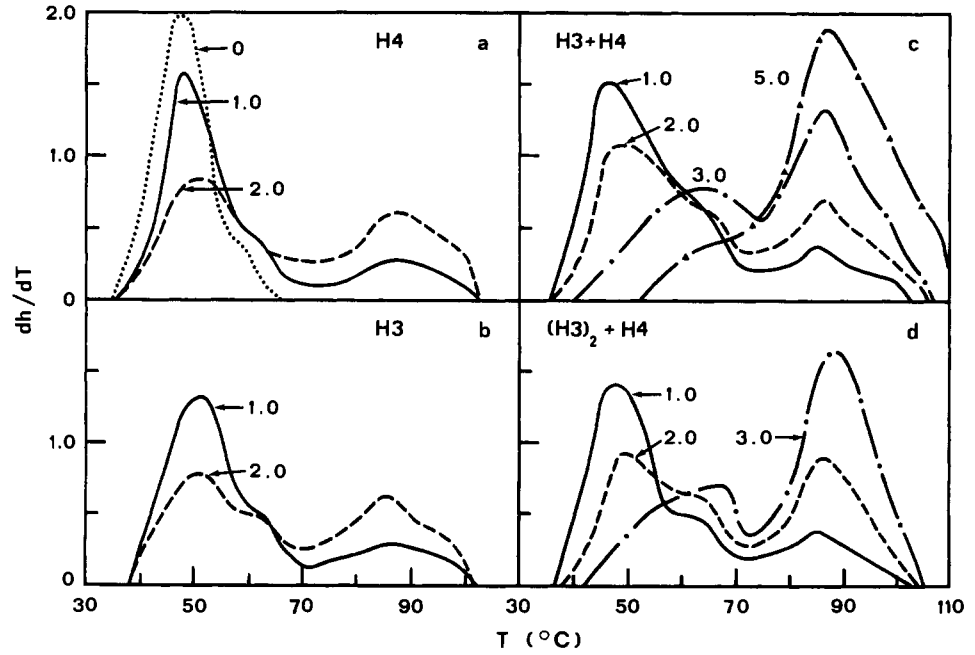

FIGURE 6: Derivative melting profiles of calf thymus histone-DNA complexes made by direct mixing in 0.01 M phosphate, pH 7.0, but dialyzed to EDTA buffer through an intermediate step of 0.1 M NaCl. (a) H4-DNA complexes; (b) H3-DNA complexes; (c) (H3 + H4)-DNA complex; (d) [$(H3)_2$ + H4]-DNA complexes. *r* value of each complex is indicated.

when [$(H3)_2$ + H4] was used, the molar concentration of the dimer, $(H3)_2$, is only one-half of that of H4. In other words, in terms of monomeric unit, the ratio of H3 to H4 is still 1:1.

For histone H3 or H4 alone, in addition to the melting bands at 47–50 and 85–87 °C, there is some broad melting between 60 and 75 °C. In directly mixed complexes in EDTA buffer, the intermediate broad melting is missing (Figure 3). As will be shown in the following paper (Yu et al., 1976), these differences could possibly be explained as being due to different conformations of histones before complex formation, whether in EDTA buffer or in phosphate buffer.

For the mixture, (H3 + H4) or [$(H3)_2$ + H4], the results are even more interesting. With these complexes in which the r value varied from 1.0 to 3.0, a substantial shoulder or even a distinguishable band appears at 60–65 °C. This is true no matter whether reduced H3 without disulfide bond or dimer $(H3)_2$ with disulfide bond(s) was used.

Because of the overlapping between the lowest melting band (47 °C) and the intermediate band (60–65 °C), the analysis of these melting curves can be only an approximation. The melting curves were roughly divided into two regions, the one for free DNA (T_m) at 47–50 °C and the rest of melting regions for histone-bound DNA (T_m'), including both 60–65 and 85–88 °C. Using this approximation, eq 2 can be used to calculate the β values of these complexes. The results are summarized in Table I. It is seen that in histone-bound regions, there are about 4.0 amino acid residues/nucleotide in the complexes, when either histone H3 or H4 was used alone, while this value becomes 3.1 for the mixture (H3 + H4) or [$(H3)_2$ + H4]. These latter values are close to the 3.0–3.5 observed for chromatin (Li, 1973a; Li et al., 1973). In other words, quantitatively, the melting results of complexes using a mixture of H3 and H4 or $(H3)_2$ and H4 are very close to those of native chromatin.

According to Figure 6c at r = 5.0, there is an initial reduction of the intermediate melting band followed by an increase of the highest melting band. The phenomenon could possibly be explained in a manner similar to that used for interpretation of polylysine binding to chromatin (Li et al., 1973). After the saturation of free DNA regions available for histone binding, the more basic regions of excess histones might still bind the DNA regions already bound by the less basic regions of histones, perhaps in the opposite groove.

Discussion

Comparison of Methods for Complex Formation. Because of the cationic property of histones at neutral pH, ionic binding is the primary interaction between histones and DNA, although hydrophobic interaction, hydrogen bonding, and other types of interaction could also be involved. If a histone molecule and DNA are directly mixed at low ionic strength, ionic binding is expected to be the determining factor for the type of complexes formed. The theoretical basis for using NaCl gradient dialysis without urea (Huang et al., 1964) or with urea (Bekhor et al., 1969; Huang and Huang, 1969) is to reduce this nonspecific ionic binding and to provide conditions for other types of interaction to play roles in binding which presumably are more specific than ionic interaction. The reason for using continuous NaCl gradient rather than stepwise gradient (Li and Bonner, 1971; Carroll, 1972) is that the former covers only several concentrations depending upon the number of steps used.

The NaCl gradient dialysis with urea (Bekhor et al., 1969; Huang and Huang, 1969) has been extensively used for reconstitution of chromatin and nucleohistones. Using this method for making reconstituted complexes, characteristic melting properties of chromatin have been successfully reproduced in histones (H2A + H2B) (Li and Bonner, 1971), less successfully with histone H2B alone (Leffak et al., 1974), and not too well with histone H4 (Shih and Bonner, 1970). In the case of histone H3, H4, or H3 + H4, using the melting properties of native chromatin as criteria for characterizing the complexes, the method of direct mixing in phosphate (method D) is better than direct mixing in EDTA (method C) which, in turn, is still better than NaCl gradient dialysis with or without urea (methods A and B). This is quite surprising since it is generally assumed that NaCl gradient dialysis with urea is the best method for making the complexes.

As to be shown in the following report (Yu et al., 1976), those complexes with better melting properties also show more negative CD near 220 nm which is closer to that in native chromatin. The formation of ordered secondary structures in histones seems to be critical for the formation of complexes resembling native chromatin.

The poor complexes formed between DNA and histone H3 or H4 using gradient dialysis with urea could possibly be explained as a result of destruction of ordered secondary structures in these histones by urea during the processes of complex formation. On the other hand, although arginine-rich histones could potentially form ordered structures in NaCl without urea, they could also form nonspecific aggregates before binding to DNA. If this explanation is correct, the results in Figures 1 and 2 suggest that, in the absence of urea, histones H3 and H4 perhaps interact with DNA (compare Figures 2c and 2d). This conclusion is in agreement with the report from Rubin and Moudrianakis (1975). The criteria used in this report and the following one (Yu et al., 1976), however, are more stringent since, in addition to the assessment of the amount of binding presented in the report of Rubin and Moudrianakis (1975), melting properties of histone-bound regions and the CD of both bound DNA and bound histones (Yu et al., 1976) are considered as criteria.

Histone H3–DNA Complexes. Except for minor variations, calf thymus histone H3 dimers containing disulfide bond(s) yield DNA complexes with melting properties similar to those of complexes formed with the H3 monomer which has no disulfide bond, regardless of what method is used for making the complexes. The implication is that, under those experimental conditions used in this report, the histone H3 dimer, with or without disulfide bond, is a fundamental subunit. Since the disulfide bond occurs in the C-terminal region, two H3 molecules would likely align themselves in parallel as opposed to antiparallel arrangements. Parallel dimer formation could further be facilitated by interaction of the more hydrophobic regions of the C-terminal halves of H3 molecules. A similar conclusion can be reached with histone H3 monomer and dimers from duck erythrocytes.

Although the primary sequence of duck histone H3 has not been published, similarity in the complexes formed with DNA suggests that the sequences of histone H3 molecules are very similar whether derived from calf thymus or from duck erythrocytes. If they do vary, the variations might not be critical as far as their binding properties with DNA are concerned. Perhaps this has some evolutionary significance with respect to structural roles of histone H3 in chromatin.

Interaction between Arginine-Rich Histones and DNA at Low Ionic Strength. As to be shown later (Yu et al., 1976), histones H3 and H4 possess some ordered structures in EDTA buffer. When bound to DNA in the same buffer, only one melting band at T_m' (87–90 °C) is induced in the complex

(Figure 3), which is close to the melting band at 82 °C in chromatin, assigned to DNA bound by the more basic regions of histones (Li and Bonner, 1971). In addition, the β value in histone-bound regions obtained in these complexes varies from 5.2 amino acid residues/nucleotide for histone H4–DNA to 6.5 for histone H3–DNA, which are roughly twice the 3.0–3.5 in native chromatin (Li, 1973a; Li et al., 1973). Both facts are in agreement with the suggestion that, when complexed directly in EDTA buffer, only the more basic regions of arginine-rich histones are tightly bound with DNA, while the less basic regions are either loosely bound or stay outside of DNA.

If the complexes were first made in 0.01 M phosphate and then dialyzed to EDTA buffer, the intermediate melting band around 60–65 °C becomes apparent for those formed from histone H3 + H4 or $(H3)_2$ + H4. According to the assignment of melting band in native chromatin (Li and Bonner, 1971; Li et al., 1973), the above results suggest that the presence of phosphate in the medium facilitates the formation of both secondary structure, as to be shown by CD in the next report (Yu et al., 1976), and subunit between histone H3 and H4 such that the less basic regions of histone can also bind DNA properly. This could be true because the β value determined (Table I) for these complexes is 3.1 amino acid residues/nucleotide which is close to the 3.0–3.5 observed for chromatin (Li, 1973a; Li et al., 1973). Even for complexes made with histone H3 or H4 alone, there is some intermediate melting around 60–65 °C and the β value is 4.0. Although the complexes formed with histone H3 or H4 alone are not better than those formed with an equimolar ratio of histones H3 and H4, they are still better than those complexes formed directly in EDTA buffer or prepared by NaCl gradient dialysis with or without urea. These facts are attributed to the formation of α-helical structures in these histones (presumably in the less basic or hydrophobic regions) in phosphate buffer (Li et al., 1972; D'Anna and Isenberg, 1974a).

It has been reported (D'Anna and Isenberg, 1974c) that an equimolar mixture of histones H3 and H4 in 0.01 M phosphate, pH 7.0, forms a subunit of tetramer. The results presented in this report, therefore, imply that a tetramer of H3 and H4 can bind DNA in a manner similar to that in native chromatin. It further implies that the formation of correct histone subunits (quaternary structure) is probably a critical condition for the formation of correct histone–DNA complexes, such as in histone H2A + H2B (Li and Bonner, 1971; Leffak et al., 1974) and histones H3 + H4 (Kornberg, 1974; Li, 1975). Indeed, histone subunits in solution or in chromatin have been observed recently (Kornberg and Thomas, 1974; Roark et al., 1974; D'Anna and Isenberg, 1974b; Martinson and McCarthy, 1975).

Since the results are identical whether a reduced histone H3 or an oxidized histone H3 was used, it is likely that a parallel histone H3 dimer, with or without disulfide bond, is a fundamental subunit in the tetramer.

A parallel dimer in histone H4 has also been suggested (Li, 1973b), based upon the consideration of sequence (DeLange et al., 1969; Ogawa et al., 1969), kinetic analysis of CD and fluorescence results (Li et al., 1972), and nuclear magnetic resonance studies (Boublik et al., 1970). Recent reports on nuclear magnetic resonance studies on histone H4 (Pekary et al., 1975a) and fragments of H4 (Pekary et al., 1975b) are in agreement with this model. Therefore, the results in this report seem to imply further that a tetramer of H3 and H4 (Kornberg and Thomas, 1974) is likely to be formed from two parallel dimers, $(H3)_2$ and $(H4)_2$, and a fundamental subunit in chromatin (Kornberg, 1974; Li, 1975).

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

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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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