Helix–Coil Transition in Nucleoprotein–Chromatin Structure†

Hsueh Jei Li,* Catherine Chang, and Manuel Weiskopf

ABSTRACT: Thermal denaturation of calf thymus nucleohistone, partially dehistonized nucleohistones and their complexes with polylysine has been studied. In 2.5 × 10^{-4} M EDTA (pH 8.0) the derivative plots of the melting profiles of native or partially dehistonized nucleohistones show three melting bands at 47° (I), 72° (III), and 82° (IV), respectively, and a shoulder near 57° (II). Polylysine binding to these nucleohistones adds a new melting band, which varies from 95 to 101° depending upon the histone content and the input ratio of lysine to nucleotide. In calf thymus nucleohistone 79 ± 3% DNA base pairs are bound by histones and there are 3.7 ± 0.4 amino acids per nucleotide in histone-bound regions. In nucleohistone, the preference for polylysine binding follows the order of free DNA regions > nonhistone protein-bound > less basic half histone-bound > more basic half histone-bound region. Polylysine strongly binds the base pairs originally bound by the less basic halves of histone molecules and it binds weakly to those originally bound by the more basic halves. The model that polylysine binds superimposed on the base pairs bound by the less basic halves of histones is favored over the model that polylysine replaces these half-molecules and binds the base pairs alone. The hyperchromicity of polylysine-bound regions also varies from 20 to 29 depending upon the protein content of the nucleohistone.

Chromatin in higher organism is composed of DNA, histones, nonhistone proteins, and RNA (Bonner *et al.*, 1968; Stellwagen and Cole, 1969; Hearst and Botchan, 1970; DeLange and Smith, 1971; Huang, 1971; Elgin *et al.*, 1971). The control of gene expression in the chromatin conceivably involves the interactions among these chromosomal components.

Recently interactions between histones and DNA have been extensively studied by physical methods. Thermal denaturation has been extensively used for investigating interactions between basic polypeptides and DNA (Tsuboi *et al.*, 1966; Leng and Felsenfeld, 1966; Olins *et al.*, 1967, 1968) or between histones and DNA (Ohlenbusch *et al.*, 1967; Olins, 1969; Shih and Bonner, 1970; Li and Bonner, 1971; Ansevin and Brown, 1971; Ansevin *et al.*, 1971; Li, 1972, 1973; Li *et al.*, 1972). Recently we have used thermal denaturation to study the interaction between histone or polylysine and DNA in terms of base pairs (Li and Bonner, 1971; Li, 1972, 1973) and to examine the binding of polylysine to histone-bound regions in chromatin (Li *et al.*, 1972).

This report extends our previous work using thermal denaturation to examine the structure of chromatin, and to provide data for the establishment of a general and self-consistent theory of helix–coil transition in nucleoprotein. In particular thermal denaturation of calf thymus nucleohistone and salt-treated nucleohistones is used for measuring both the fraction of DNA base pairs bound by histones and the average number of amino acids per nucleotide in histone-bound regions of calf thymus chromatin. Polylysine binding to these nucleohistones has also been studied with the aim of probing the distribution of electrostatic charges along the chromatin molecule.

Materials and Methods

Calf thymus chromatin was prepared according to the method of Shih and Bonner (1969). The soluble and the salt-treated nucleohistones were prepared from chromatin as previously described (Li and Bonner, 1971). They were dialyzed against 2.5 × 10^{-4} M EDTA (pH 8.0) for the thermal denaturation and the polylysine binding studies.

Calf thymus DNA was purchased from Sigma Chemical Co. and was purified by phenol extraction. The molar extinction coefficient of 6500 M^{-1} cm^{-1} in nucleotide was used for both nucleohistones and DNA.

Poly(L-lysine) hydrochloride (mol wt 170,000) was purchased from Schwarz/Mann. It was dialyzed against the EDTA buffer. Since a change in concentration may occur after dialysis, the polylysine concentration in EDTA buffer of each stock solution used for titration with DNA or nucleohistones was determined by the ninhydrin method (Spies, 1957), using lysine hydrochloride as the standard. Using this method, we noticed that the concentration of the polylysine stock solution used for the preparation of a polylysine–nucleohistone complex which we reported earlier (Li *et al.*, 1972) was 30% lower than the reported value. The preparation of polylysine–DNA or polylysine–nucleohistone complexes by direct and slow mixing of polylysine and DNA or nucleohistone has previously been described (Li *et al.*, 1972). After centrifugation at 10,000 rpm in a Sorvall SS-34 rotor at 4°, the supernatant was collected. The absorbance and thermal denaturation measurements of the supernatant solutions were obtained using a Gilford spectrophotometer Model 2400-S. The derivative plots dh/dT of the melting curves are reported as we did before (Li and Bonner, 1971). h is the per cent increase in hyperchromicity referred to the absorbance A_{260} at 260 nm at room temperature. The heating rate was about $^2/_3$°/min. Thermal denaturation solutions were in a buffer of 2.5 × 10^{-4} M EDTA at pH 8.0. All the samples have negligible light scattering with a A_{320}/A_{260} ratio less than 0.04.

Results

Thermal Denaturation of Chromatin, Sheared Chromatin, and Nucleohistone. Our recent reports on the mechanism of thermal denaturation of nucleoproteins (Li and Bonner, 1971; Li, 1972, 1973; Li *et al.*, 1972) were based upon a simple hypothesis that thermal stability of DNA base pairs in a nucleo-

† From the Department of Chemistry, Brooklyn College of the City University of New York, Brooklyn, New York 11210. *Received October 31, 1972.* Supported by National Science Foundation Grant GB35459 and Research Foundation of The City University of New York. M. W. was a recipient of a National Science Foundation Undergraduate Summer Research award.

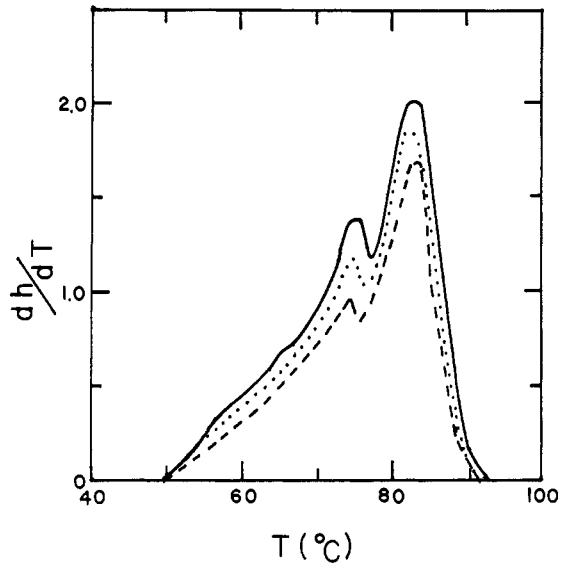

FIGURE 1: Derivative melting profiles of purified chromatin (– – –), sheared chromatin (. . . .), and nucleohistone (——). h_{max} is 25.2 for purified chromatin, 29.1 for sheared chromatin and 33.3 for nucleohistone.

protein is primarily determined by an irreversible ionic interaction between phosphates on DNA and basic amino acids on proteins. It is a primary interaction and is the most important factor for the stability of DNA base pairs. Secondary interactions, such as aggregation among nucleoprotein molecules into larger particles, folding of a large nucleoprotein molecule, the formation of supercoil in chromatin etc., may also have some significant effects on the thermal stability of the DNA. This hypothesis was tested by determining the thermal denaturation characteristics of purified chromatin, sheared chromatin, and soluble nucleohistone. Purified chromatin was the pellet of crude chromatin after sucrose gradient centrifugation (Shih and Bonner, 1969). It is readily pelleted at 10,000 rpm for 10 min in a Sorvall SS-34 rotor. Purified chromatin was sheared in a Waring blender for 2 min and centrifuged at 10,000 rpm for 30 min in a Sorvall SS-34 rotor. The supernatant is the sheared chromatin. Nucleohistone is the pellet of sheared chromatin after sucrose gradient centrifugation at 36,000 rpm for 24 hr in SW41 rotor.

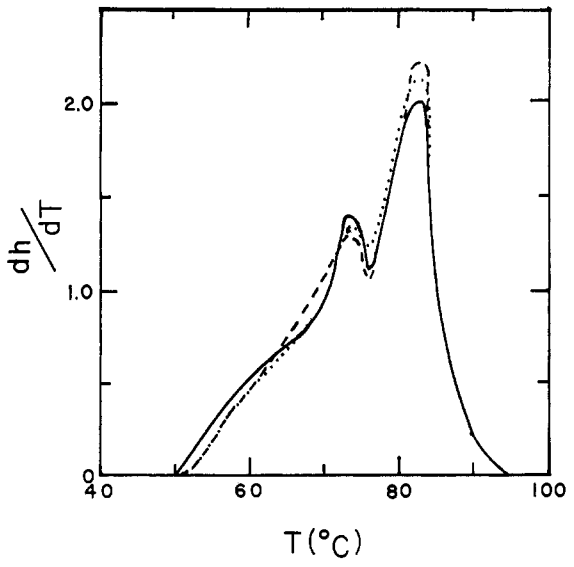

FIGURE 2: Normalized derivative melting profiles of purified chromatin (– – –), sheared chromatin (. . . .), and nucleohistone (——). h_{max} of 33.3 is used for all three curves.

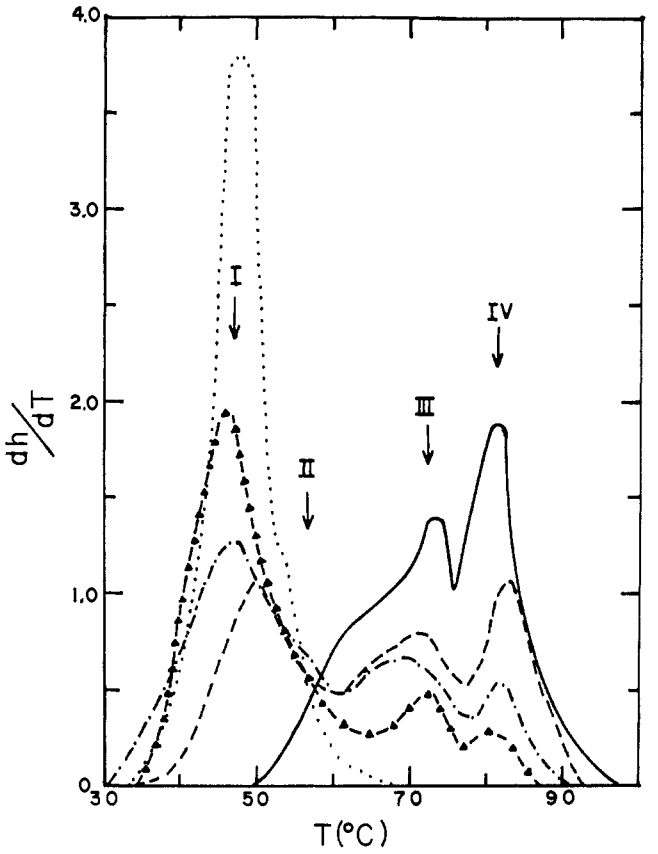

FIGURE 3: Derivative melting profiles of NaCl-treated nucleohistones. NaCl is 0.0 M (——), 0.6 M (– – –), 1.6 M (–·–), 2.5 M (–▲–), and DNA (. . . . .).

Figure 1 shows the derivative plots of the melting profiles of chromatin, sheared chromatin and nucleohistone. It appears that there are quantitative differences. The results in Figure 1 indicate that thermal denaturation of nucleoprotein may depend upon the size of the molecule. However, the results in Figure 2 exclude this possibility. The maximum hyperchromicity, h_{max}, in Figure 1 is 33.3 for nucleohistone, 29.1 for sheared chromatin, and 25.2 for chromatin. This decrease in hyperchromicity is due to the contribution of light scattering to the recorded absorbance at 260 nm, since there is an increase in the A_{320}/A_{260} ratio at room temperature, of 0.023 for nucleohistone, 0.056 for sheared chromatin and 0.135 for chromatin. The light-scattering contribution to A_{260} apparently raises the measured absorbance and consequently reduces the hyperchromicity on melting. Normalizing the h_{max} of the samples to 33.3, the h_{max} of nucleohistone, gives the data shown in Figure 2. Within experimental error, they are identical with one another. This result implies that the size of chromatin, before or after shearing, has no significant effect on its melting property.

Thermal Denaturation of Nucleohistone and NaCl-Treated Nucleohistones. Melting profiles of calf thymus nucleohistone and salt-treated nucleohistones have been reported before by Ohlenbusch *et al.* (1967). The derivatives of the melting profiles are shown in Figure 3. They are similar to our previous

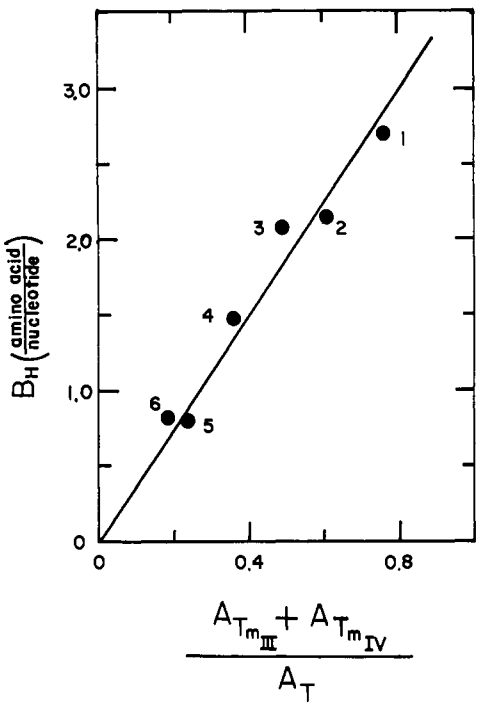

FIGURE 4: Linear relation between histone content and the area of melting band. Nucleohistones are, respectively, pretreated by NaCl of M (1), 0.35 M (2), 0.60 M (3), 1.0 M (4), 1.6 M (5), and 2.5 M (6).

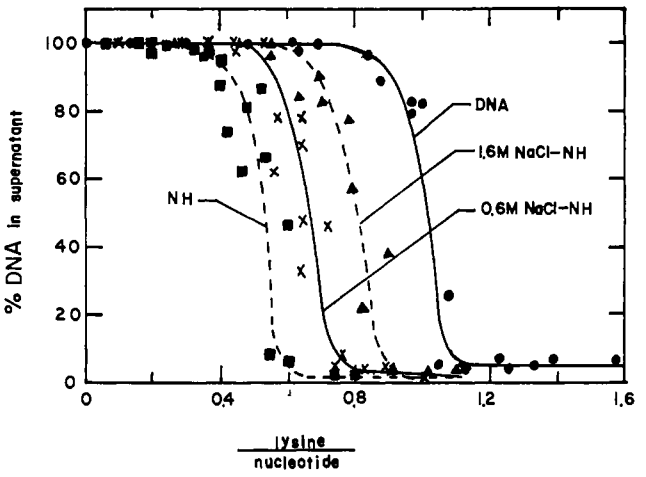

FIGURE 5: Titration curves of nucleohistones by polylysine. Nucleohistone (■), 0.6 M NaCl-treated nucleohistone (×), 1.6 M NaCl-treated nucleohistone (▲), and DNA (●).

data for pea bud nucleohistones (Li and Bonner, 1971; Li, 1972), which were dissociated by high NaCl concentrations. This resulted in a decrease in the melting bands at 72 and 82° and was accompanied by an increase in the free DNA melting band at 47°. As was done previously, the four melting bands are assigned. Melting band I at 47° corresponds to free DNA regions. Melting band II or the shoulder near 57° corresponds to DNA regions bound by nonhistone proteins or short free DNA gaps between histone-bound regions or both. Melting band III at 72 ± 2° and IV at 82 ± 2° correspond respectively to DNA regions bound by the less basic and the more basic half-molecules of histones.

The melting temperature ($T_{m,I}$) of free DNA regions is sensitive to ionic strength (Li and Bonner, 1971) and varies as much as ±5° when EDTA buffers from various preparations are used (Shih and Bonner, 1970). The concentrations of NaCl required to diminish equivalent amounts of higher melting bands in this report are generally higher than those values reported by Ohlenbusch *et al.* (1967).

Since melting bands III and IV are due to the melting of DNA base pairs bound by histones (Li and Bonner, 1971), the fraction of the area of these two melting bands can be used for the measurement of the fraction of DNA base pairs bound by histones (Li, 1973). If the hyperchromicity of DNA base pairs are independent of protein binding, which is approximately true in nucleohistones (Ohlenbusch *et al.*, 1967; Li, 1973), then the following equation is obeyed (Li, 1973)

$$B_k = \beta_k \frac{A_k}{A_T} \qquad (1)$$

where B_k is the total amino acids of protein k per nucleotide, A_k is the area under melting band k, and A_T the total melting area which is equal to h_{max}. The slope β_k is the average number of amino acids per nucleotide in those DNA regions bound by protein k.

In nucleohistones, if B_H is the total amino acids of histones per nucleotide in native or NaCl-treated nucleohistones, eq 1 becomes

$$B_H = \beta_H \left[\frac{A_{Tm,III} + A_{Tm,IV}}{A_T} \right] \qquad (2)$$

β_H is the average number of amino acids per nucleotide in histone-bound regions in nucleohistone. B_H, histone content in nucleohistone or salt-treated nucleohistones, was determined by acid extraction (0.5 N H_2SO_4) as previously described, (Li, 1973). The results are shown in Figure 4 and they fit eq 2. The average slope from five preparations of nucleohistones and NaCl-treated nucleohistones is 3.7 ± 0.4 amino acids per nucleotide compared with 3.2 in pea bud nucleohistone and 2.9 to 3.3 in nucleohistone I (Li, 1973).

The fraction of melting bands III and IV

$$\frac{A_{Tm,III} + A_{Tm,IV}}{A_T}$$

is a measure of the fraction of DNA base pairs bound by histones. These fractions of native calf thymus nucleohistones were determined as 76, 82, 84, 79, and 74%, with an average of 79 ± 3%, in five preparations. Thus, 79 ± 3% of DNA base pairs are bound by histones in calf thymus nucleohistone, compared with 75 ± 8% in pea bud nucleohistone (Li, 1973).

Polylysine Binding to DNA, Nucleohistone, and NaCl-Treated Nucleohistones. A slow addition of polylysine to DNA results in irreversible complexes with polylysine scattered along the DNA molecule (Tsuboi *et al.*, 1966). The complexes are soluble until a certain lysine/nucleotide ratio is reached (Clark and Felsenfeld, 1971). Figure 5 shows polylysine titration curves of calf thymus nucleohistone, 0.6 M NaCl- and 1.6 M NaCl-treated nucleohistones and DNA. In each case the complexes are completely soluble until a characteristic ratio of lysine/nucleotide is reached. Precipitation then occurs and is complete in a small range of lysine/nucleotide ratios. The midpoint of titration or precipitation is respectively 0.52, 0.66, 0.81, and 1.02 for native, 0.6 and 1.6 M NaCl-treated nucleohistones and DNA. When more histones are dissociated from

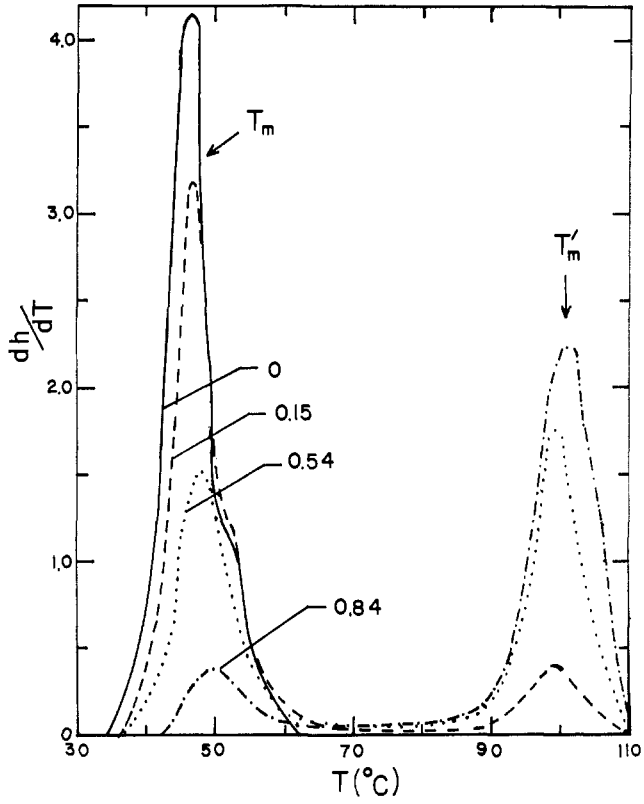

FIGURE 6: Derivative melting profiles of polylysine–DNA complexes. Lysine/nucleotide ratios are given in the figure.

DNA, more DNA base pairs become available for polylysine binding. The titration curves of nucleohistones and DNA are in agreement with those reported by Clark and Felsenfeld (1971).

Figure 6 shows some typical results of thermal denaturation of polylysine–DNA complexes. Two special features can be seen in these data. First the melting band of pure DNA is not symmetrical with respect to the peak. There is a shoulder at the higher temperatures which may indicate the existence of either heterogeneous DNA molecules of different GC contents or clusters of GC-rich and AT-rich regions along calf thymus DNA molecule (Crothers, 1968). Second, if the melting temperature of each melting band is defined as the temperature of the peak, free DNA regions have a T_m slightly shifted from 47 to 50° when more polylysine binds to DNA. Similar increases in the T_m of free DNA regions has been observed in basic polypeptide–DNA complexes (Olins *et al.*, 1967, 1968) and histone–DNA complexes (Olins, 1969; Shih and Bonner, 1970; Li and Bonner, 1971). The T_m shifts have been explained as a result of the melting of shorter free DNA segments (Li and Bonner, 1971; Li, 1972).

T_m', the melting temperature of polylysine-bound regions in the complex, also shifts slightly from 99 to 101° when lysine/nucleotide ratio is increased from 0.15 to 0.84. A similar shift in T_m' was observed for polylysine–DNA complexes prepared by salt gradient dialysis (Olins *et al.*, 1967).

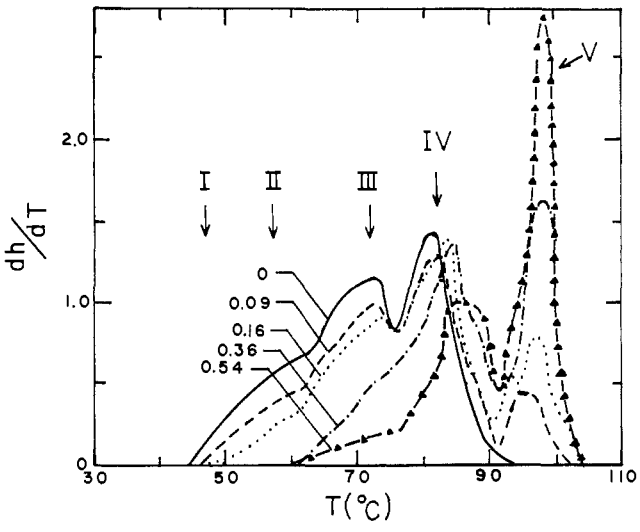

FIGURE 7: Derivative melting profiles of polylysine–nucleohistone complexes. Lysine/nucleotide ratios are given in the figure.

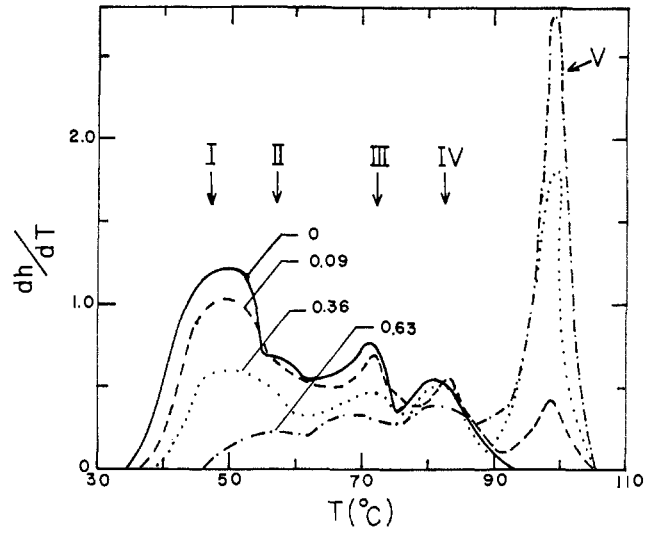

FIGURE 8: Derivative melting profiles of polylysine–1.6 M NaCl-treated-nucleohistone complexes. Lysine/nucleotide ratios are given in the figure.

Some typical results of thermal denaturation of polylysine–nucleohistone and polylysine–1.6 M NaCl-treated nucleohistones are shown in Figures 7 and 8. In addition to the four melting bands (I, II, III, and IV) (Figure 3), there is a new melting band (V) at 95–99° corresponding to the melting of polylysine-bound regions. Qualitatively it can be seen that when more polylysine binds to nucleohistone or salt-treated nucleohistones the areas of melting bands I, II, and III decrease. The area of melting band IV is not significantly decreased until a high lysine/nucleotide ratio is reached. However, a gradual shift of $T_{m,IV}$ to a higher temperature is noticed as the lysine/nucleotide ratio is increased. In polylysine–nucleohistone (Figure 7) or polylysine–0.6 M NaCl-treated nucleohistone (not presented in this report), $T_{m,V}$ of polylysine-bound regions is increased from 95 to 98° when the lysine/nucleotide ratio is increased from about 0.1 to about 0.6. Nucleohistone treated by 1.6 M NaCl has more free DNA regions and its $T_{m,V}$ (98–99°) is closer to the T_m' (99–101°) of the polylysine–DNA complex.

Preference Sites on Nucleohistone for Polylysine Binding. Polylysine is essentially a polycation which carries one positive charge per lysine residue under our experimental condition. Nucleohistone can be regarded as a polyanion with one negative charge per nucleotide which is partially neutralized by histones. According to our previous experiments (Li and

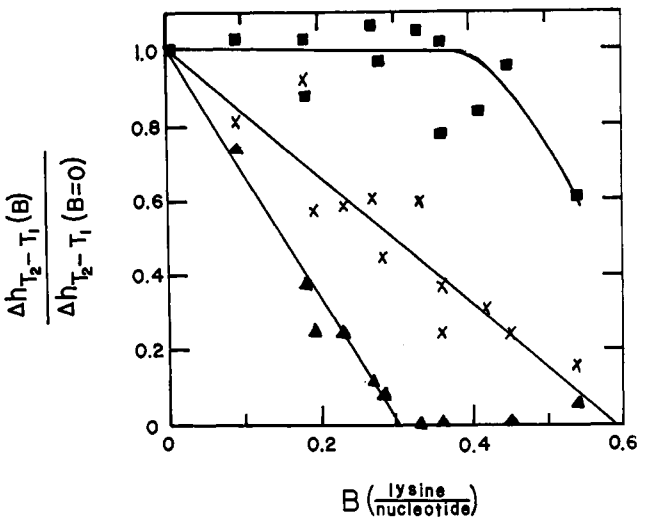

FIGURE 9: Decrease of hyperchromicity as a function of lysine/nucleotide ratio in polylysine–nucleohistone complexes. $T_2 - T_1$ is 60° − 50° (▲), 75° − 65° (×), and 86° − 78° (■). For $B = 0$, $\Delta h_{60-50°}$ is 3.6, $\Delta h_{75-65°}$ is 9.6, and $\Delta h_{86-78°}$ is 9.7.

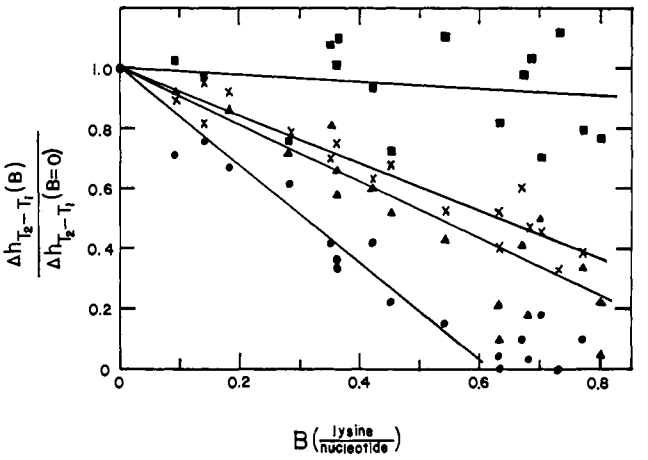

FIGURE 10: Decrease of hyperchromicity as a function of lysine/nucleotide ratio in polylysine–1.6 M NaCl-treated–nucleohistone complexes. $T_2 - T_1$ is 50° − 30° (○), 60° − 50° (▲), 75° − 65° (×), and 86° − 78° (■). For $B = 0$, $\Delta h_{50-30°} = 16.9$, $\Delta h_{60-50°} = 6.3$, $\Delta h_{75-65°} = 4.5$, and $\Delta h_{86-78°} = 2.9$.

Bonner, 1971; Li, 1972) melting band I corresponds to the melting of free DNA regions, band II to DNA regions bound by nonhistone proteins or very short free DNA gaps between two adjacent histone-bound regions, and bands III and IV to DNA regions bound by the less basic and the more basic half-molecules of histones. Simply based upon electrostatic interaction it would be expected that the order of preference sites for polylysine binding will be free DNA > non-histone protein-bound > less basic half histone-bound > more basic half histone-bound. Qualitatively this order of preference is supported by the data in Figures 7 and 8. In order to obtain a quantitative comparison the results in Figures 7 and 8 are analyzed as follows.

In the presence of polylysine the resolution of a melting curve into individual melting bands becomes difficult (Figures 7 and 8). Therefore we use the differences of hyperchromicity near the peaks of each melting band as a measurement of the fraction of DNA base pairs of that particular melting band not bound by polylysine. We define $\Delta h_{T_2-T_1} = h(T_2) - h(T_1)$, where $h(T)$ is the hyperchromicity of the complex at temperature T. $\Delta h_{50-30°}$ is used for measuring melting band I, $\Delta h_{60-50°}$ for band II, $\Delta h_{75-65°}$ for band III, and $\Delta h_{86-78°}$ for band IV. Taking these values of the control (lysine/nucleotide = 0) as 1.0, the fractions of these values in the presence of polylysine provide quantitative measurements of the fractions of DNA base pairs of each melting band which are still free of polylysine binding. The results are shown in Figures 9 and 10. For nucleohistone (Figure 9), $\Delta h_{60-50°}$ and $\Delta h_{75-65°}$ decrease linearly as the lysine/nucleotide ratio is increased. $\Delta h_{60-50°}$ decreases faster than $\Delta h_{75-65°}$. $\Delta h_{60-50°}$ is totally diminished at lysine/nucleotide = 0.3 while $\Delta h_{75-65°}$ is totally eliminated at lysine/nucleotide = 0.6. $\Delta h_{86-78°}$ is constant until lysine/nucleotide is larger than 0.4 when precipitation starts to occur. In 1.6 M NaCl-treated nucleohistone (Figure 10) the decrease in the hyperchromicity difference follows the order of $\Delta h_{50-30°} > \Delta h_{60-50°} > \Delta h_{75-65°}$. $\Delta h_{86-78°}$ decreases only slightly and has a large experimental error because the $\Delta h_{86-78°}$ for the control is as small as 2.9. Similar results are also observed for 0.6 M NaCl-treated nucleohistone. The results here indicate that the preference for polylysine binding follows the order of free DNA ($\Delta h_{50-30°}$) > non-histone protein-bound ($\Delta h_{60-50°}$) > less basic half histone-bound ($\Delta h_{75-65°}$) > more basic half histone-bound ($\Delta h_{86-78°}$).

Spectral Changes in Thermally Denatured Polylysine–DNA and Polylysine–Nucleohistone Complexes. Based upon the absorbance at 260 nm, all the complexes with lysine/nucleotide ratio below 0.90 are soluble and stay in the supernatant after centrifugation (Table I). The constant absorbance value of the DNA base pairs when the lysine/nucleotide ratio is equal to or smaller than 0.90 indicate that polylysine–DNA complexes and pure DNA have the same molar extinction coefficient at 260 nm. Figure 11 shows molar absorption spectra of pure DNA and polylysine–DNA complex (lysine/nucleotide = 0.90) in the native state (ϵ_N), at 30°, in the denatured state (ϵ_D) at 110° and the differences $\epsilon_D - \epsilon_N$.

Before melting, the polylysine–DNA complex, compared with DNA, has a slightly higher absorbance between about 300 nm to about 270 nm and a much higher absorbance below 240 nm. Presumably, the latter is contributed by the absorbance of polylysine in the complex. The absorption spectrum of the melted complex, however, is noticeably different from that of pure DNA (Figure 11). This difference becomes clearer in the difference spectra $\epsilon_D - \epsilon_N$. After denaturation polylysine–DNA complex has a smaller hyperchromicity than pure DNA. In both cases, polylysine–DNA complex or pure

TABLE I: Hyperchromicities of Polylysine–DNA Complexes.

Lysine/ Nucleotide	A_{260}[a] in Supernatant	h_{max} (%)	A_{320}/A_{260} 30°	A_{320}/A_{260} 110°
0	0.648	35.9	0.022	0.016
0.09	0.645	35.8	0.019	0.022
0.18	0.647	34.9	0.009	0.007
0.36	0.653	32.5	0.031	0.023
0.63	0.640	32.0	0.037	0.039
0.90	0.669	30.0	0.035	0.034

[a] The volume change after adding polylysine has been corrected.

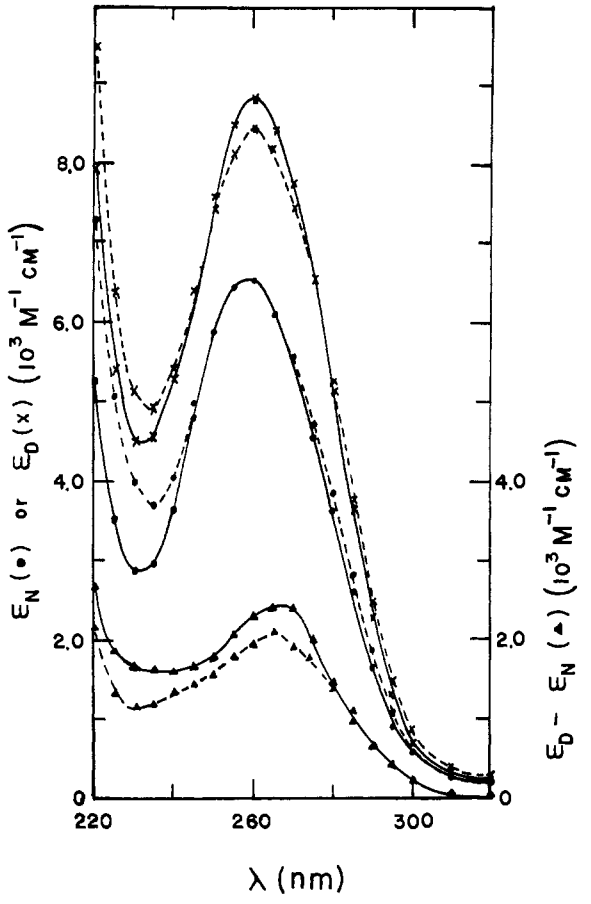

FIGURE 11: Absorption and difference spectra of pure DNA and polylysine–DNA complex before and after thermal denaturation. Pure DNA (——) and polylysine–DNA complex with 0.9 lysine/nucleotide (– – – – –).

DNA, the hyperchromicity has its maximum at 265 nm rather than at 260 nm, the peak of the absorption spectra.

Within experimental error, h_{max} is decreased when more DNA base pairs are bound by polylysine (Table I). It is reduced from 35.9 to 30% when the lysine/nucleotide ratio is increased from 0 to 0.90. Such a reduction of hyperchromicity is not due to light scattering since the A_{320}/A_{260} ratio varies only from 0.01 to 0.04 in these complexes. Further there is no significant change in the A_{320}/A_{260} ratio of each complex before and after melting.

A similar reduction in hyperchromicity was observed in the polylysine–nucleohistone complexes (Table II) and polylysine–NaCl-treated nucleohistones.

Quantitative Analysis of Polylysine Binding to Nucleohistone, NaCl-Treated Nucleohistones, and DNA. Since the hyperchromicity of polylysine-bound regions are lower than that of polylysine-free regions (Tables I and II) eq 1 cannot be used because it is based upon the assumption that all the base pairs in the complexes have the same hyperchromicity no matter whether they exist in free DNA regions or in protein-bound regions. In order to analyze the results of the complexes presented here the following equations will be used.

Let h_b, h_f, A_b, and A_f, be respectively the hyperchromicities (h) and the areas of melting bands (A) of base pairs bound by and free of polylysine. In polylysine–nucleohistone complexes, A_b is equal to A_V of melting band V. In polylysine–DNA complexes, A_b is equal to $A_{T_m'}$. If A_T is the total melting area

TABLE II: Hyperchromicities of Polylysine–Nucleohistone Complexes.

Lysine/ Nucleotide	A_{260}[a] in Supernatant	h_{max}	A_{320}/A_{260} 30°	A_{320}/A_{260} 110°
0	0.650	33.5	0.030	0.023
0.09	0.650	32.7	0.029	0.023
0.18	0.654	31.9	0.023	0.020
0.23	0.640	31.5	0.021	0.022
0.27	0.638	30.7	0.020	0.018
0.36	0.635	29.4	0.022	0.020
0.45	0.608	27.8	0.035	0.027

[a] The volume change after adding polylysine has been corrected.

$$A_T = A_b + A_f \tag{3}$$

If F is the fraction of base pairs tightly bound by polylysine which melt at temperature corresponding to melting of A_b, $1 - F$ will be the fraction of base pairs free of polylysine. We then obtain

$$A_b = h_b F \tag{4}$$

$$A_f = h_f(1 - F) \tag{5}$$

Let B be the input ratio of lysine per nucleotide. Before the binding is saturated, there is a linear relation between B and F. We can write

$$B = \beta F \tag{6}$$

where β is the average number of lysine per nucleotide in polylysine-bound regions (Li, 1973). Equation 4 becomes

$$B = \frac{\beta}{h_b} A_b \tag{7}$$

The slope of the linear plot of B against A_b is β/h_b. Combining eq 5 and 6 we obtain

$$B = \beta\left(1 - \frac{A_f}{h_f}\right) \tag{8}$$

The linear plot of B against $[1 - (A_f/h_f)]$ gives β from which we can determine h_b from eq 7. Since B, h_f, A_f, and A_b can be determined experimentally, the parameters β and h_b can be determined from eq 7 and 8.

Experimentally, h_f was taken as the hyperchromicities of nucleohistone, salt-treated nucleohistones or DNA in the absence of polylysine binding. A_b in polylysine–DNA complexes, or in polylysine–nucleohistone complexes was measured directly from graphs such as Figures 6–8. A_f is simply equal to $A_T - A_b$, where A_T, the total area, is equal to h_{max}.

Figure 12 presents the results of the plot of eq 8 of the complexes of polylysine with native, 0.6 and 1.6 M NaCl-treated nucleohistones and DNA. The straight lines of different slopes are not readily apparent from the figure because of some scattered points in each complex. Nevertheless, they are the

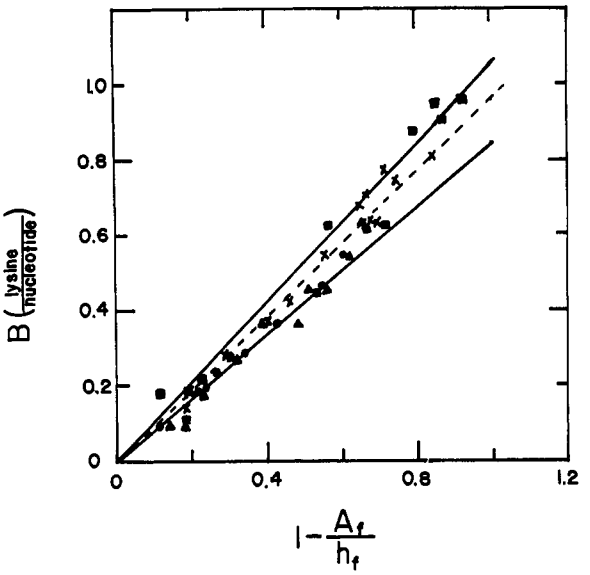

FIGURE 12: Linear plot of eq 8. Nucleohistone (●), 0.6 M NaCl-treated nucleohistone (▲), 1.6 M NaCl-treated nucleohistone (x), and DNA (■).

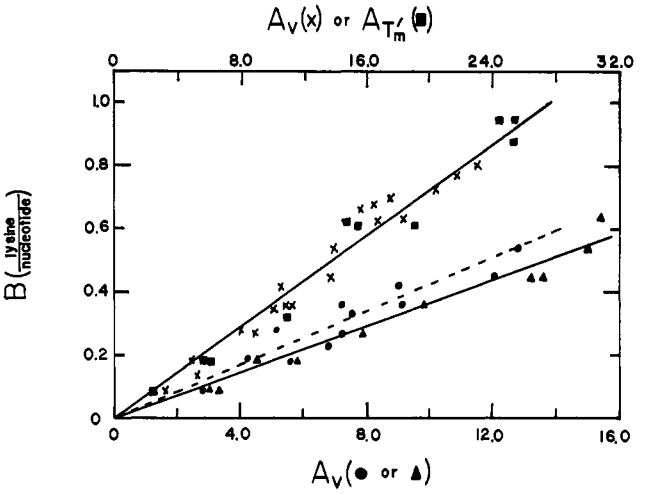

FIGURE 13: Linear plot of eq 7. Nucleohistone (●), 0.6 M NaCl-treated nucleohistone (▲), 1.6 M NaCl-treated nucleohistone (x), and DNA (■).

best straight lines we could draw when the data of each complex were plotted separately. The slope is 0.85 for polylysine–nucleohistone and polylysine–0.6 M NaCl-treated nucleohistone complexes, 0.95 for polylysine–1.6 M NaCl-treated nucleohistone complexes and 1.05 for polylysine–DNA complexes. Thus, the apparent average number of lysine residues per nucleotide in polylysine-bound regions is increased from 0.85 to 1.05 when the protein content (histone and nonhistone proteins) is decreased from that in nucleohistone to zero in pure DNA.

The results of the plot of eq 7 are given in Figure 13. The slope, β/h_b, is 0.0425 for nucleohistone, 0.0366 for 0.6 M NaCl, 1.6 M NaCl-treated nucleohistones, and pure DNA. Using the values of β determined in Figure 12, h_b is determined to be 20.0 for nucleohistone, 23.2 for 0.6 M NaCl-treated nucleohistone, 26.0 for 1.6 M NaCl-treated nucleohistone, and 28.7 for pure DNA. The hyperchromicity of DNA base bairs tightly bound by polylysine depends upon whether these pairs are also bound by histones and/or nonhistone proteins. The significance of the variations in β and h_b will be discussed later.

Discussion

Thermal Denaturation and Primary Interactions in Nucleoprotein. The primary interactions between proteins and DNA are used here to describe direct interactions between the protein molecule and certain DNA base pairs complexed with protein. Several possible interactions, such as ionic bonding, hydrogen bonding, and hydrophobic forces, can be involved. These interactions can cause changes in the conformation of both proteins and the double helix of the DNA segment which is directly complexed by the proteins. We assume that only these primary interactions between proteins and DNA base pairs have a significant effect on the melting of the base pairs. Secondary interactions do not appear to affect the melting properties of nucleoproteins, although they may participate in extensive structural changes in the molecule. For example, if many protein-bound regions do exist on the same DNA molecule a regular structure such as a supercoil in the whole nucleoprotein molecule may be induced. It is also possible that two or more nucleoprotein molecules can link to one another through the proteins such as in chromosomes. If the nucleoprotein is very long, folding of the molecule is also possible and folding may actually occur more easily in nucleoprotein than in DNA because of the presence of proteins. It might therefore be anticipated that secondary interactions such as those just mentioned may have some effect on thermal denaturation of nucleoproteins.

The results in Figure 2 exclude the possibility that the size of the chromatin molecule has an effect on melting, since sheared chromatin and nucleohistone which are much smaller in size than chromatin have the same melting curves. However, if the DNA is as short as 100 base pairs, the unwinding of both ends of DNA becomes significant and the resulting end effect slightly lowers the T_m of pure DNA (Crothers *et al.*, 1965) and the T_m' of polylysine–DNA complexes (Olins *et al.*, 1968).

There is evidence showing that DNA base pairs are tilted in chromatin, which is gradually restored to the B form when histones are removed (Permogorov *et al.*, 1970; Simpson and Sober, 1970; Shih and Fasman, 1970). Nevertheless, characteristic melting bands corresponding to histone binding (melting bands III and IV) do not disappear when more histones are removed by higher salts (Li and Bonner, 1971; Li, 1972). Thus, thermal denaturation of a nucleoprotein is not sensitive to its higher order structures. It simply reflects the primary interactions between proteins and DNA base pairs. A recent report of Shih and Lake (1972), showing that metaphase and interphase chromatins of Chinese Hamster cells have identical melting curves although morphologically they appear differently, supports this hypothesis.

Chromatin Structure. An important question concerning the binding of histones to DNA in chromatin is what length of DNA is bound by each histone molecule. Since the binding of polylysine or polyarginine to DNA has 1 to 1 stoichiometry (one basic residue per nucleotide) (Tsuboi *et al.*, 1966; Leng and Felsenfeld, 1966; Olins *et al.*, 1967, 1968; Shih and Bonner, 1970), it might be expected that every basic residue on histones binds one phosphate on DNA with complete charge neutralization on the histone-bound regions. This is not sup-

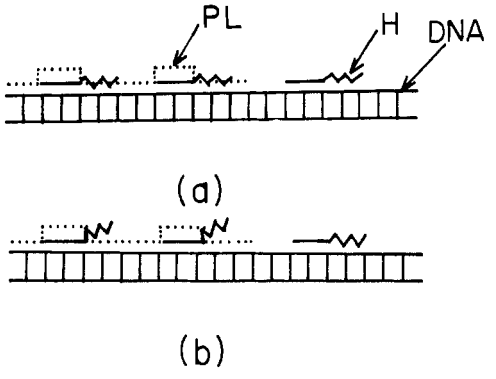

FIGURE 14: Two models of polylysine binding to nucleohistone. Polylysine has a molecular weight of about 170,000 and covers many histone-bound segments. H is histone and PL is polylysine. The more basic halves of histones (straight line) and the less basic halves of histone (squiggly line). Model a suggests superimposed binding of polylysine to the less basic halves of histones. Model b suggests a replacement of the less basic halves of histones by polylysine.

ported by the findings that electrostatic shielding on melting band III is less than that of melting band IV which is less than that of polylysine–DNA complexes (Li and Bonner, 1971). The large differences in melting temperatures among these melting bands, $T_{m,III} = 72°$ and $T_{m,IV} = 82°$ in nucleohistone and $T_m' = 100°$ in polylysine–DNA complex, also argue against this hypothesis.

The results in this report and in the previous one (Li, 1973) indicate that in the histone-bound regions of chromatin there are about 3.5 amino acid residues per nucleotide or 7 amino acid residues per base pair. We would then expect that histone IIb2 with 125 amino acid residues (Iwai *et al.*, 1970) binds 18 base pairs; that histone IV with 102 amino acid residues (DeLange *et al.*, 1969; Ogawa *et al.*, 1969) binds 15 base pairs and histone I with 216 amino acid residues (Bustin *et al.*, 1969) binds 31 base pairs. The number of nucleotides bound by each histone molecule are approximately equal to the numbers of basic residues in that molecule. Our results indicate less electrostatic shielding on histone-bound regions of nucleohistones than in the polylysine–DNA complex. It implies that some basic residues in histones do not directly interact with phosphates on DNA. Another attempt to study histone binding to DNA by using Corey–Pauling–Koltum (CPK) molecular model and the sequences of histones has been reported by Shih and Bonner (1970).

Based upon the findings in pea bud nucleohistones we previously proposed that when a histone molecule binds certain base pairs on DNA in the chromatin, the more basic half-molecules of histones stabilize base pairs at higher temperature (melting band IV at 82°) and the less basic half at a lower temperature (melting band III at 66° in pea bud or 72° in calf thymus) (Li and Bonner, 1971; Li, 1972). Our results favor the view that both half-molecules of histones bind DNA base pairs and they reject the view that only the more basic ends bind DNA while the less basic ends are free. The results from calf thymus as presented here confirm our previous conclusion which is also supported by the findings of Ansevin *et al.* (1971). These authors elegantly showed that trypsin treatment of chromatin reduces the highest melting band, corresponding to our $T_{m,IV}$, more than the second highest melting band, which corresponds to our $T_{m,III}$. This is in agreement with our interpretation of the two melting bands because trypsin specifically cleaves peptide bonds at lysine and arginine residues. The more basic half-molecules of histones will be more rapidly hydrolyzed than the less basic halves. Thus, in this case, DNA base pairs originally bound by the more basic halves will be released more readily from histone binding than those bound by the less basic halves.

Our results in this report also favor the previous interpretation of melting bands III and IV. Since polylysine binding to chromatin or DNA is essentially electrostatic, those DNA regions with more phosphates available for the binding will have a preference for polylysine. Our results show that preference follows the order of free DNA regions (melting band I) > non-histone protein-bound (melting band II) > less basic half histone-bound (melting band III), > more basic half histone-bound (melting band IV). These results agree with our previous findings that electrostatic shielding follows the opposite order (Li and Bonner, 1971) and they agree also with our interpretation of melting bands III and IV.

To what extent are DNA base pairs in chromatin bound by histones? Our results here indicate that 79 ± 3% of DNA base pairs in calf thymus chromatin are bound by histones. In pea bud it is 75 ± 8% (Li, 1973). These results indicate that about 20–25% of DNA base pairs in chromatin are free of histone binding which includes free DNA regions as well as those base pairs bound by non-histone proteins. This is in agreement with the values obtained by template activity or DNA–RNA hybridization methods (Marushige and Bonner, 1966; Bonner *et al.*, 1968; Gilmour and Paul, 1969; Shih and Bonner, 1970; Kurashina *et al.*, 1970). It disagrees with the conclusion that 50% of DNA in chromatin is free as reported by Clark and Felsenfeld (1971). Their conclusion was based upon the nuclease digestion experiments and upon the polylysine titration of chromatin. Using nuclease digestion as a measurement of free DNA regions may be inadequate. Mirsky (1971) showed that the amount of DNA in chromatin digested by DNase depends on both the amount of enzyme added and the time of enzyme action. Itzhaki (1971) also showed release of proteins accompanied with DNase digestion of chromatin. Using polylysine titration for measuring free DNA regions is not appropriate either because polylysine binds not only free DNA regions but also DNA base pairs originally bound by nonhistone proteins or histones.

A further question about chromatin structure is that concerned with the size of free DNA regions in chromatin. It was shown previously that formaldehyde fixation enhances the dependence of T_m on the sizes of free DNA regions and can thus be used for a qualitative comparison of the sizes of free DNA regions (Li, 1972). However, a quantitative relation between the shift in T_m and its size is still lacking and therefore a quantitative estimation of the sizes of free DNA regions in chromatin by the thermal denaturation method is still not possible. Nevertheless, the existence of significant amounts of very long stretches of free DNA regions in chromatin can be excluded because there is no significant amount of melting at $T_{m,I}$ in chromatin (Figures 2 and 3). Similar results on melting of chromatin have also been observed before (Ohlenbusch *et al.*, 1967; Paoletti and Huang, 1969; Ansevin and Brown, 1971; Li and Bonner, 1971).

Theoretical models of chromosome structure in higher organisms have recently been proposed (Crick, 1971; Paul, 1972). Crick (1971) proposed a possible structure of globular DNA with a twisted hairpin constructed from part of a double-stranded DNA and a single-stranded DNA loop serving as recognition sites for control purposes. Single-stranded DNA with base stacking melts at a lower temperature than double-stranded DNA and it certainly will melt at a

much lower temperature than histone-protected double-stranded DNA. So far no melting band lower than $T_{m,I}$, the melting temperature of free DNA regions, has been detected in chromatin. Therefore, it can be concluded that such single-stranded DNA loops, if they exist at all, constitute not more than a few per cent of the total DNA genome, the amount that can be detected by hyperchromicity measurement. The structural role of histones, proposed by Crick (1971), that they bridge two adjacent or opposite segments of a double-helical double helix (as illustrated in his Figure 3), is not consistent with our proposal that both halves of each histone molecule bind neighboring regions of DNA (Li and Bonner, 1971; Li, 1972).

Polylysine Binding to DNA and Nucleohistone. It is interesting to note that adding polylysine to DNA or nucleohistone does not change the molar absorbance of DNA, indicating that only a minor structural change on DNA or nucleohistone results from polylysine binding. It is strikingly different from polylysine–DNA complexes prepared by salt gradient dialysis. These reconstituted complexes always show strong light scattering (Olins *et al.*, 1967, 1968; Kawashima *et al.*, 1969; Shih and Bonner, 1970; Carroll, 1972) and big optical changes as measured by circular dichroism (Haynes *et al.*, 1970; Carroll, 1972). Further, because of negligible light scattering, the hyperchromic shift of our polylysine–DNA complexes can be accurately measured and significant decreases are observed (Table I). In this case modified eq 7 and 8 rather than eq 1 should be used for the calculation. Whether there are similar decreases in the hyperchromicity of reconstituted polylysine–DNA complexes is not known.

In polylysine–nucleohistone complexes a question was raised before (Li *et al.*, 1972) whether a polylysine molecule binds the less basic halves of histones or simply replaces these regions and binds directly on DNA base pairs. With our present quantitative data we are able to say something about this question.

Figure 14a,b show two different models of binding of polylysine to nucleohistone. In both models polylysine does not bind strongly to the DNA base pairs bound by the more basic halves of histones. This conclusion is deduced from the results of Figures 7–10. Model a shows that polylysine binds superimposed upon the less basic halves of histones while model b shows the replacement of these halves of histones by polylysine.

The average number of lysine per nucleotide, β, in polylysine-bound regions is decreased from 1.05 in DNA to 0.85 in nucleohistone. Two factors should be considered in these numbers. Those lysine residues which hang over the regions bound by the more basic halves of histones are not counted in melting band V because they only shift $T_{m,IV}$ slightly instead of producing a new band at $T_{m,V}$. If β_{real} is the real average number of lysine per nucleotide in polylysine-bound regions corresponding to $T_{m,V}$ in nucleohistone, we would expect that $\beta > \beta_{real}$, because those lysine residues hung over the more basic halves of histones are counted in β but not in β_{real}. In model b we assume that polylysine-bound regions in nucleohistone ($T_{m,V}$) are the same as those in pure DNA (T_m'), we would then expect that β_{real} equal 1.05, the value obtained from polylysine–DNA complexes. This contradicts the expectation that β_{real} should be less than β which is 0.85 in nucleohistone. On the contrary, model a implies that polylysine binding to the less basic halves of histones needs much less lysine per nucleotide because some phosphates in these regions have already been bound or neutralized partially by the basic residues in histones. β_{real} in model a would be much lower than 1.05, the value in polylysine–DNA complexes. So far, we have no criterion for estimating β_{real} in model a. Nevertheless, the expectation that β_{real} is much lower than 1.05 does not contradict the finding that $\beta = 0.85$ in nucleohistone. The consideration of β values therefore favors model a over b.

The result that the apparent hyperchromicity, h_b, of DNA base pairs bound by polylysine is decreased from 28.7 in DNA to 20.0 in native nucleohistone also favors model a over b. We would expect the same h_b for these complexes if model b were the right one.

References

Ansevin, A. T., and Brown, B. W. (1971), *Biochemistry 10*, 1133.

Ansevin, A. T., Hnilica, L. S., Spelsberg, T. C., and Kehn, S. L. (1971), *Biochemistry 10*, 4793.

Bonner, J., Dahmus, M. E., Fambrough, D., Huang, R. C. C., Marushige, K., and Tuan, D. Y. H. (1968), *Science 159*, 47.

Bustin, M., Rall, S. C., Stellwagen, R. H., and Cole, R. D. (1969), *Science 163*, 391.

Carroll, D. (1972), *Biochemistry 11*, 421.

Clark, R. J., and Felsenfeld, G. (1971), *Nature (London), New Biol. 229*, 101.

Crick, F. (1971), *Nature (London) 234*, 25.

Crothers, D. M. (1968), *Biopolymers 6*, 1391.

DeLange, R. J., Fambrough, D. M., Smith, E. L., and Bonner, J. (1969), *J. Biol. Chem. 244*, 319.

DeLange, R. J., and Smith, E. L. (1971), *Annu. Rev. Biochem. 40*, 279.

Elgin, S. C. R., Froehner, S. C., Smart, J. E., and Bonner, J. (1971), *Advan. Cell Mol. Biol. 1*, 1.

Haynes, M., Garrett, R. A., and Gratzer, W. B. (1970), *Biochemistry 9*, 4410.

Hearst, J., and Botchan, M. (1970), *Annu. Rev. Biochem. 39*, 151.

Huang, P. C. (1971), *Progr. Biophys. Mol. Biol. 23*, 103.

Itzhaki, R. F. (1971), *Biochem. J. 125*, 221.

Iwai, K., Ishikawa, K., and Hayashi, H. (1970), *Nature (London) 226*, 1056.

Kawashima, S., Inour, S., and Ando, T. (1969), *Biochim. Biophys. Acta 186*, 145.

Kurashina, Y., Ohba, Y., and Miguno, D. (1970), *J. Biochem. (Tokyo) 67*, 661.

Leng, M., and Felsenfeld, G. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1325.

Li, H. J. (1972), *Biopolymers 11*, 835.

Li, H. J. (1973), *Biopolymers* (in press).

Li, H. J., and Bonner, J. (1971), *Biochemistry 10*, 1461.

Li, H. J., Chang, C., and Weiskopf, M. (1972), *Biochem. Biophys. Res. Commun. 47*, 883.

Marushige, K., and Bonner, J. (1966), *J. Mol. Biol. 15*, 160.

Mirsky, A. E. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2945.

Ogawa, Y., Quagliaroti, G., Jordan, J., Taylor, C. W., Starbuck, W. C., and Busch, H. (1969), *J. Biol. Chem. 244*, 4387.

Ohlenbusch, H. H., Olivera, B. M., Tuan, D., and Davidson, N. (1967), *J. Mol. Biol. 25*, 229.

Olins, D. E. (1969), *J. Mol. Biol. 43*, 439.

Olins, D. E., Olins, A. L., and von Hippel, P. H. (1967), *J. Mol. Biol. 24*, 157.

Olins, D. E., Olins, A. L., and von Hippel, P. H. (1968), *J. Mol. Biol. 33*, 265.

Paoletti, R. A., and Huang, R. C. C. (1969), *Biochemistry 8*,

1615.
Paul, J. (1972), *Nature (London) 238*, 444.
Paul, J., and Gilmour, R. S. (1966), *J. Mol. Biol. 16*, 242.
Permogorov, U., Debabov, I. U. G., Sladkova, I. A., and Rebentish, B. A. (1970), *Biochim. Biophys. Acta 199*, 556.
Shih, T. Y., and Bonner, J. (1969), *Biochim. Biophys. Acta 182*, 30.
Shih, T. Y., and Bonner, J. (1970), *J. Mol. Biol. 48*, 469.
Shih, T. Y., and Fasman, G. D. (1970), *J. Mol. Biol. 52*, 125.
Shih, T. Y., and Lake, R. S. (1972), *Biochemistry 11*, 4811.
Simpson, R. B., and Sober, H. (1970), *Biochemistry 9*, 3103.
Spies, J. R. (1957), *Methods Enzymol. 3*, 467.
Stellwagen, R. H., and Cole, R. D. (1969), *Annu. Rev. Biochem. 38*, 951.
Tsuboi, M., Matsuo, K., and Tso, P. O. P. (1966), *J. Mol. Biol. 15*, 256.

Scission of *Escherichia coli* Deoxyribonucleic Acid in Alkali†

Walter E. Hill‡ and Walton L. Fangman*

ABSTRACT: DNA released from *Escherichia coli* spheroplasts exhibits an apparent decrease in molecular weight when centrifuged in alkaline sucrose gradients for long times (low rotor speeds) compared to shorter times (higher rotor speeds). Most of this decrease in molecular weight can be accounted for by alkali-catalyzed scission of DNA strands during centrifugation. At 0.3 N NaOH, *E. coli* DNA in crude lysates is cleaved at a rate of about one break/10^9 daltons per hr. λ closed duplex DNA rings are cleaved at a similar rate. Prolonged exposure of high molecular weight cellular DNAs to alkali will result in cleavage and an underestimation of single-strand molecular weights.

A common procedure for determination of the molecular weight of DNA single strands involves treatment of duplex DNA at alkaline pH. It has been known for a long time that, whereas RNA is rapidly hydrolyzed under these conditions, DNA appears to be quite stable. Nevertheless, there is evidence that alkali produces scissions in DNA, albeit at a slow rate. Purines are lost from the DNA backbone at alkaline pH (Greer and Zamenhof, 1962), and these sites are subsequently hydrolyzed (Shapiro and Chargaff, 1964). During experiments concerned with the effect of DNA synthesis inhibitors on *Escherichia coli* DNA molecular weight, we observed that DNA sedimented in alkaline sucrose gradients exhibited an apparently lower molecular weight when centrifuged for longer times. In this report we show that most of this effect results from alkaline cleavage of DNA strands. Rates of scission of DNA in alkali are high enough to be consequential in studies on the chromosomal DNA molecules of bacteria and eukaryotic cells which are 10^9 daltons and larger.

Experimental Procedure

Bacteria and Culture Conditions. The bacteria were derived from *E. coli* K12SH-28, a thymidine phosphorylase negative (Tpp$^-$) strain (Fangman, 1969): FA220 (Tpp$^-$, Arg$^-$, Lac$^-$, Tdr$^-$ (thymidine requiring)), FA210 (Tpp$^-$, λ$^+$ lysogen). All experiments were carried out with aerobic exponential phase cultures at 37°. For experiments with bacterial DNA, FA220 was grown in F buffer (Sadler and Novick, 1965) supplemented with 4 mg/ml of glucose, 2 mg/ml of decolorized Casamino Acids, and 2 μg/ml of [*methyl*-^{3}H]thymidine (50 μCi/ml) for at least three generations. Covalently closed λ DNA duplexes (form I) were produced by infecting strain FA210 with ^{3}H-labeled λ CI857 at a multiplicity of infection of 10 and incubating for 90 min (Fangman and Feiss, 1969).

Zone Sedimentation. On-gradient lysis of cells was carried out by a procedure similar to that of McGrath and Williams (1966). Approximately 3×10^8 cells (FA220 or λ-infected FA210) were suspended in 0.15 ml of 0.125 M sucrose–0.05 M EDTA–0.005 M Tris (pH 8). After addition of 0.04 ml of 1% lysozyme, samples were incubated 15 min at 37°. Spheroplasts were either layered directly onto sucrose gradients or lysed by addition of an equal volume of NaOH solution and incubated at 25°. Twenty-five microliters of material were layered onto 5–20% sucrose gradients (5 ml) containing either 0.3 N NaOH–0.7 M NaCl or 0.1 N NaOH–0.9 M NaCl. After 10 min at room temperature, the tubes were centrifuged in a Spinco SW50L rotor at 25°. With this rotor a speed of 10,000 rpm produces a centrifugal force of 8161*g* at the midpoint of the sample tube. Fractions collected from the bottom of the tubes were made 5% in trichloroacetic acid, and the precipitate washed on glass fiber filters with cold 5% trichloroacetic acid and 95% ethanol. Gradients contained at least 7000 cpm of [^{3}H]DNA.

[^{3}H]DNA released from λ virus particles by incubation with 1% sodium dodecyl sulfate for 30 min at 37° was employed as a sedimentation standard. Number-average molecule weight (M_n) of *E. coli* DNA was calculated as $M_n = 1/\Sigma(c_i/m_i)$, where c_i = fraction of total counts per minute in a gradient fraction and m_i = the molecular weight of DNA in that fraction (Fangman and Russel, 1971). Molecular weights were calculated from the equation of Studier (1965) for sedimentation in alkali. The number of breaks per 10^9 daltons of *E. coli* DNA was calculated as: $(1 \times 10^9$ daltons/observed $M_n) - 1$.

The fraction of ^{3}H-labeled λ DNA existing as form I was determined (Fangman and Feiss, 1969) by centrifuging samples through sucrose gradients containing 0.3 N NaOH–0.7 M NaCl at 32,000 rpm for 55 min (25°). The amount of unbroken form I

† From the Department of Genetics, University of Washington, Seattle, Washington 98195. *Received August 14, 1972.* Supported by National Science Foundation Research Grant GB-19792 and the National Institute of Health Predoctoral Training Grant GM-00182.

‡ Present address: Laboratory of Molecular Biology, University of Wisconsin, Madison, Wis. 53706.