# Interaction of Histone Half-Molecules with Deoxyribonucleic Acid*

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ABSTRACT: Derivative plots of the melting profiles of native or partially dehistonized pea bud nucleohistones in $2.5 \times 10^{-4}$ M EDTA (pH 8.0) show three melting bands at 42° (I), 66° (III), and 81° (IV), respectively, and a shoulder near 52° (II). The positions of the bands are independent of what kinds or how many histones are removed from DNA. They are therefore characteristic properties of nucleohistone. The melting bands at 66° (III) and at 81° (IV) are both due to histone stabilization of DNA. The melting band at 42° (I) is due to free DNA segments while that at 52° is in part due to nonhistone protein binding and in part to weak coupling of melting at the boundaries between free DNA and histone-bound DNA segments as well as to the small gaps between adjacent histone-bound segments. The effect of increased ionic strength is greatest on the $T_m$ of melting band I, less on that of melting band III and least on that of melting band IV. This indicates qualitatively that electrostatic shielding of the phosphates of DNA by histone is greatest for the segments of melting band IV, less for those of melting band III and least for those of melting band I. The completeness of renaturation of DNA of segments of melting band III depends on the intactness of the segments of melting band IV. This suggests that the DNA segments of melting bands III and IV are physically connected. Histone II–DNA complexes reconstituted by continuous salt gradient dialysis in the presence of urea exhibit melting bands at 66 and 80°, thus similar to those of native nucleohistone. Histone IIb2-N-terminal half-molecules with 0.32 basic amino acid/residue stabilize DNA to a $T_m$ of 70° while IIb2-C-terminal half-molecules with 0.20 basic amino acids per residue stabilize only to 57°. Thus the two ends of this typical histone molecule stabilize DNA to different degrees.

Deoxyribonucleic acid when complexed with histone in the form of nucleohistone is not available for transcription (Huang and Bonner, 1962; Barr and Butler, 1963; Allfrey *et al.*, 1963). The histone–DNA complex is therefore of interest. The interaction between histones and DNA has been investigated intensively (Zubay and Doty, 1959; Bonner and Ts'o, 1964; Huang *et al.*, 1964; Johns and Butler, 1964; Akinrimisi *et al.*, 1965; Ohlenbusch *et al.*, 1967; Olins, 1969; Shih and Bonner, 1970a). Because it is difficult to prepare pure histones, basic polypeptides as polylysine and polyarginine have also been used as models for the study of basic protein–DNA interaction (Akinrimisi *et al.*, 1965; Tsuboi *et al.*, 1966; Leng and Felsenfeld, 1966; Olins *et al.*, 1967, 1968; Shih and Bonner, 1970b). We concern ourselves only with histones.

Thermal denaturation profiles have been a major tool for studies of nucleoproteins. In the case of basic polypeptide–DNA complexes, melting profiles exhibit biphasic transitions if the ratio of cation to anion is less than 1 (Tsuboi *et al.*, 1966; Olins *et al.*, 1967, 1968; Shih and Bonner, 1970b). However, melting profiles of native nucleohistones are generally broad (Ohlenbusch *et al.*, 1967), and are difficult to analyze. We have found derivative plots of melting profiles more amenable to such analysis.

A thermal transition appears as a band in the derivative plot of a melting profile. A DNA–macromolecule complex such as nucleohistone will therefore exhibit as many melting bands as it exhibits thermal phase transitions.

Derivative plots of melting profiles have been used earlier (Gould and Simpkins, 1969; Riesner *et al.*, 1969; Shih and Bonner, 1970a). We use the technique for the more complicated system of native nucleohistone. It will be shown below that nucleohistone possesses two intrinsic melting bands which are due to histone binding to DNA and that these do not depend on what kinds or how much histone has been removed from the nucleohistone. The renaturation of partially denatured nucleohistone has been examined to determine the extent of coupling between the DNA segments responsible for these two melting bands.

To further investigate the origin of the two melting bands, histone IIb2 was cleaved in the middle and the N- and C-terminal half-molecules separated. These were complexed with DNA and the effectiveness of the two ends on thermal stabilization of DNA examined. Our results suggest a model in which the more basic end of the histone stabilizes DNA more (melting band IV) and the less basic end less (melting band III).

## Materials and Methods

*Preparation of Pea Bud Nucleohistone.* Pea bud chromatin was prepared according to the methodology of Bonner *et al.* (1968). The purified chromatin, suspended in 0.01 M Tris (pH 8.0), was sheared by Virtis at 40 V for 90 sec and then centrifuged for 20 min at 16,000 rpm in the SW39 rotor. The supernatant was collected and dialyzed extensively against 0.01 M Tris (pH 8.0). The purified pea bud nucleohistone was stored in liquid nitrogen until use. Purified nucleohistone thus prepared has negligible scattering with $A_{320}/A_{260} < 0.02$.

*Salt-Treated Nucleohistone.* In each series of histone removal from nucleohistone, either by NaCl, $H_2SO_4$, or $MgCl_2$, 4 ml of purified nucleohistone in 0.01 M Tris, pH 8.0 ($A_{260} \sim 30$), was added with continual swirling to an equal volume of salt or acid of appropriate concentration, also

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buffered by 0.01 M Tris (pH 8.0). This was layered over 2.5 ml of 1.7 M sucrose and centrifuged at 45,000 rpm for 24 hr in Spinco 65 rotor. The supernatant was pipetted down to the sucrose phase and discarded. The pellet was collected and dialyzed against $2.5 \times 10^{-4}$ M EDTA (pH 8.0). These constitute salt-extracted nucleohistone.

*Cyanogen Bromide (CNBr) Cleavage of Histone IIb2.* Histone from calf thymus was kindly prepared by Miss M. Thomas. It contains IIa, IIb1, and IIb2. Histone IIb2 has methionine residues at positions 58 and 61, respectively (Iwai *et al.*, 1969); the other histones II contain no methionine. The methionine-containing peptide can be specifically cleaved by CNBr (Gross, 1967; Iwai *et al.*, 1969). To histone II (~60 mg) dissolved in 1 ml of 0.1 N HCl was added 1 ml containing an excess of CNBr also in 0.1 N HCl. After 20-hr reaction at room temperature the mixture was lyophilized to remove unreacted CNBr. It was then dissolved in 1 ml of 0.02 N HCl.

*Sephadex G-75 Column Chromatography.* CNBr-treated histone II was applied to a Sephadex G-75 column (1 × 52 cm) in 0.02 N HCl and developed with 0.02 N HCl. Histone concentration of the effluent was determined spectrophotometrically at 240 mμ.

*Amberlite CG-50 Column Chromatography.* The CNBr-cleaved histone IIb2 fraction (second peak from Sephadex G-75 fractionation) was dialyzed against 0.1 M acetic acid and lyophilized. It was then dissolved in 1–2 ml of 5% Gdm·HCl[1] buffered in 0.1 M phosphate buffer (0.05 M $NaH_2PO_4$ + 0.05 M $Na_2HPO_4$). The two peptides, histone IIb2-N (N-terminal-half) and histone IIb2-C (C-terminal-half), were separated by development with a Gdm·HCl gradient (5–30%) as described by Bonner *et al.* 1968a).

*Disc Electrophoresis.* Qualitative identification of histone IIb2-N, IIb2-C, and intact histone II was carried out by disc electrophoresis through polyacrylamide gels (Reisfeld *et al.*, 1962; Bonner *et al.*, 1968).

*Amino Acid Composition Analysis.* The amino acid compositions of purified fractions of histone IIb2-N and IIb2-C were determined with a modified Beckman–Spinco automatic amino acid analyzer, Model 120B. The analyses presented are uncorrected for loss during hydrolysis.

*Reconstitution in Continuous Salt Gradient in the Presence of Urea.* Reconstitution of histones to DNA was done by dialysis in the presence of 5 M urea as used by Shih and Bonner (1970a) and with a continuously decreasing gradient of NaCl (2.0–0.1 M) all in 5 M urea–0.01 M Tris (pH 8.0). The mixture was dialyzed from 2.0 to 0.1 M NaCl over a period of 40 hr or longer. Urea was then continuously dialyzed out in the presence of 0.015 M NaCl–0.01 M Tris (pH 8.0). The preparation was finally dialyzed against $2.5 \times 10^{-4}$ M EDTA (pH 8.0) for melting.

*Analysis.* Calf thymus DNA from Sigma Chemical Co. was purified by phenol extraction. 20.9 $A_{260}$ units/mg of DNA and 4.25 $A_{280}$ units/mg of histones were used for concentration determination (Ohlenbusch *et al.*, 1967). In some experiments the Lowry procedure (Lowry *et al.*, 1951) was also used for histone determination before and after reconstitution.

*Derivative Melting Profile.* The buffer used for melting experiments in this report is $2.5 \times 10^{-4}$ M EDTA (pH 8.0) with $Na^+ \approx 7.5 \times 10^{-4}$ M (Ohlenbusch *et al.*, 1967). A Gilford Model 2000 multiple-sample absorbance spectrophotometer equipped with recorder was used for melting at a constant heating rate of *ca.* 2/3° per min. The increased hyperchromicity, $h_{260}(T)$, referred to $A_{260}$ at 25° was recorded degree by degree. The slopes of the melting profile at temperature, $T$, was obtained by

$$\frac{dh_{260}(T)}{dT} = \frac{h_{260}(T+1) - h_{260}(T-1)}{2}$$

*Buffers with Different Ionic Strengths.* For the study of the effect of ionic strength on melting behavior of nucleohistone, buffers at the same EDTA concentration but at varied NaCl concentrations were prepared. Buffer A is the standard buffer, $2.5 \times 10^{-4}$ M EDTA (pH 8.0). Adding varied concentrations of NaCl, 0.001, 0.005, 0.01, and 0.025 M to buffer A yields buffers B, C, D, and E with ionic strengths $1.75 \times 10^{-3}$, $5.75 \times 10^{-3}$, $1.08 \times 10^{-2}$, and $2.58 \times 10^{-2}$ M, respectively.

*Denaturation and Renaturation.* Nucleohistone in buffer A was denatured to varied temperatures, 55, 65, 73, 80, or 95°, respectively, at a heating rate of 2/3° per min. In each case it was renatured slowly at a cooling rate of 1/6° per min back to room temperature. It was then again denatured completely, *i.e.*, to over 95°.

## Results

*Melting Spectra of NaCl-Treated Nucleohistone.* Pea bud nucleohistones, native or NaCl-treated, in general show broad melting profiles in buffer A ($2.5 \times 10^{-4}$ M EDTA, pH 8.0) as does calf thymus nucleohistone (Ohlenbusch *et al.*, 1967). At least three phases can be distinguished in such a melting profile, and these can be readily visualized if the derivative of the melting profile, $dh_{260}/dT$ is plotted as a function of temperature (Figure 1). The three phases appear as melting bands at 42, 66, and 81°, and a shoulder near 52°.

Histones are dissociated from nucleohistones by salt (Ohlenbusch *et al.*, 1967; Fambrough and Bonner, 1968). Thus lysine-rich histone I but no others is completely dissociated by 0.6 M NaCl. The data of Figure 1 show that when histone I is removed from nucleohistone, the melting bands at 66 and 81° are both diminished, and that at 42° increased. Removal of more histone from nucleohistone by higher NaCl concentrations results in further decrease of the bands at 66 and 81° and a further increase in that at 42°. Thus the former two bands both appear to be attributable to melting of DNA stabilized by histone and the latter to deproteinized DNA.

Results entirely similar to those of Figure 1 are obtained when histones are dissociated from nucleohistone by increasing concentrations of $MgCl_2$ or of $H_2SO_4$.

*Resolution of Derivative Melting Profile into Intrinsic Melting Bands.* The melting profile of nucleohistone is then an intrinsic property of the histone–DNA complex. Three melting bands can be distinguished, namely those at 42, 66, and 81°. If it is assumed that each melting band is symmetric with respect to its peak, then the derivative melting profile can be resolved into four melting bands with peaks at 42, 52, 66, and 81°, respectively (Figure 2). By calculating the fractional area under each melting band we are able to estimate the fraction of DNA having that specific thermal stability. Table I summarizes the partition of DNA among melting bands for nucleohistones treated by NaCl. As more histone is dissociated from nucleohistone by a higher salt

[1] Abbreviation used is: Gdm·HCl, guanidinium hydrochloride.

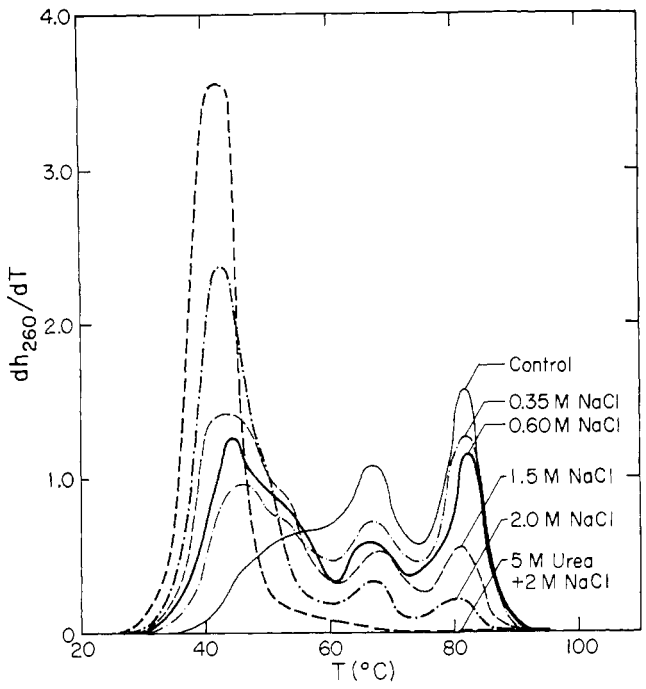


FIGURE 1: Derivative melting profiles of NaCl-treated nucleohistones in buffer A ($2.5 \times 10^{-4}$ M EDTA, pH 8.0).

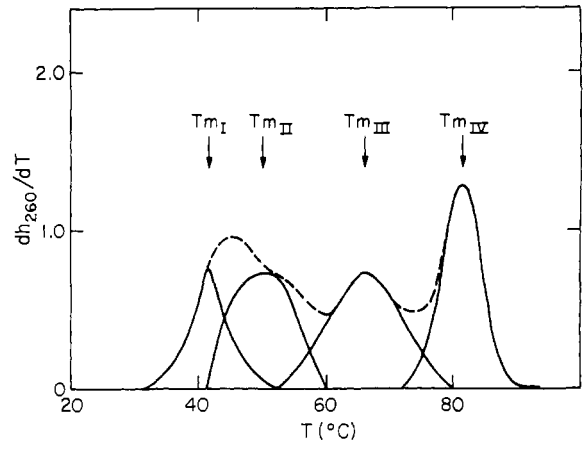


FIGURE 2: Resolution of the derivative melting profile of nucleohistone into intrinsic melting bands (0.35 M NaCl-treated nucleohistone in buffer A). ------, measured derivative melting profile; ——, melting bands. $T_{mI} = 42°$, $T_{mII} = 52°$, $T_{mIII} = 66°$, $T_{mIV} = 81°$.

concentration the fractional areas under bands III and IV decrease. This is accompanied by an increase of nearly equal amount under band I. The area under band II remains rather constant until most of the histones are dissociated, *e.g.*, by 2.0 M or higher NaCl concentration.

It is clear that melting band I (42°) is due to free DNA segments and band III (66°) and IV (81°) to histone-bound DNA segments. The source of melting band II (52°) is not so obvious. Since the bulk of nonhistone protein is not removed by 2.0 M NaCl (Fambrough and Bonner, 1968), nonhistone protein binding to DNA may contribute partly to melting band II. Short free DNA gaps between two neighboring histone-bound segments, and even the boundaries between free DNA and histone-bound DNA segments, may also contribute to this melting band. This point will be discussed in more detail below. In any case, melting band II probably results from several sources as indicated below by its heterogeneous response to ionic strength.

The data of Table I show that the areas under melting bands III and IV are nearly identical at all levels of histone removal. When histone I is selectively removed (0.6 M NaCl), the decrease in band III is more than that in band IV (Table I). Therefore histone I binding on DNA contributes more to band III than to IV. Nevertheless, histone I does contribute to both bands.

*Ionic Strength Effects on the Melting of Nucleohistone.* We have studied the effect of ionic strength on the two melting bands, III and IV, which are attributable to histone binding. Melting bands which respond similarly to increases in ionic strength will exhibit melting profiles near their $T_m$'s which are parallel to one another but shifted toward higher temperature. Figure 3a–c show the melting profiles of native, 1.0 and 3.0 M NaCl-treated nucleohistones in buffers A, B, C, and D. Parallel shifts of parts of the profiles are evident. The increase in temperature required to produce a given increase in hyperchromicity as between buffers of increasing ionic strength is plotted in Figure 4a–c. In each case $\Delta T_m$, that is the change in $T_m$ as between buffers A and B, A and C, A and D, etc., is plotted as a function of hyperchromicity. Parallel shifts of melting profiles are indicated in this plot by horizontal segments. One such region occurs at early melting which, in buffer A, is *ca.* 42° and therefore belongs to melting band I. The other two parallel shifts caused by increasing ionic strength occur during later melting which, in buffer A, correspond to the melting at 66 and 81° and are due to melting bands III and IV. The appearance of parallel shifts of melting profiles near the peaks of their melting bands indicate that the nucleohistones of each melting band, exhibit similar responses toward ionic strength. No parallel shift of melting profile with increased ionic strength occurs with melting band II. This indicates that the sources which contribute to melting band II respond heterogeneously toward ionic strength. As noted, melting band II may be due to nonhistone protein binding to DNA, to boundaries between free and histone-bound DNA segments and/or to short gaps between two adjacent histone-bound DNA segments.

Melting band I of native nucleohistone (Figure 4a) does not exhibit a parallel shift of melting profile with increasing ionic strength. NaCl (3.0 M)-treated nucleohistone also does not. This is because such small fractions of total DNA are

TABLE I: Fractions of Area under Each Melting Band (%).[a]

| Nucleohistone Treated by NaCl | $A_I$ | $A_{II}$ | $A_{III}$ | $A_{IV}$ |
|---|---|---|---|---|
| Control | 4.2 | 25.8 | 37.6 | 32.4 |
| 0.35 M | 12.4 | 32.6 | 26.7 | 27.3 |
| 0.60 M | 21.4 | 29.2 | 24.7 | 24.7 |
| 1.0 M | 25.3 | 30.2 | 22.5 | 22.0 |
| 1.5 M | 34.5 | 30.1 | 18.9 | 16.5 |
| 2.0 M | 53.6 | 25.2 | 12.7 | 8.5 |
| 3.0 M | 69.0 | 17.8 | 8.0 | 5.2 |
| 5 M urea + 2 M NaCl | 97.0 | 3.0 | 0 | 0 |

[a] Each derivative melting profile of Figure 1 was resolved into four melting bands. $A_i$ is the percentage of area under the i band.

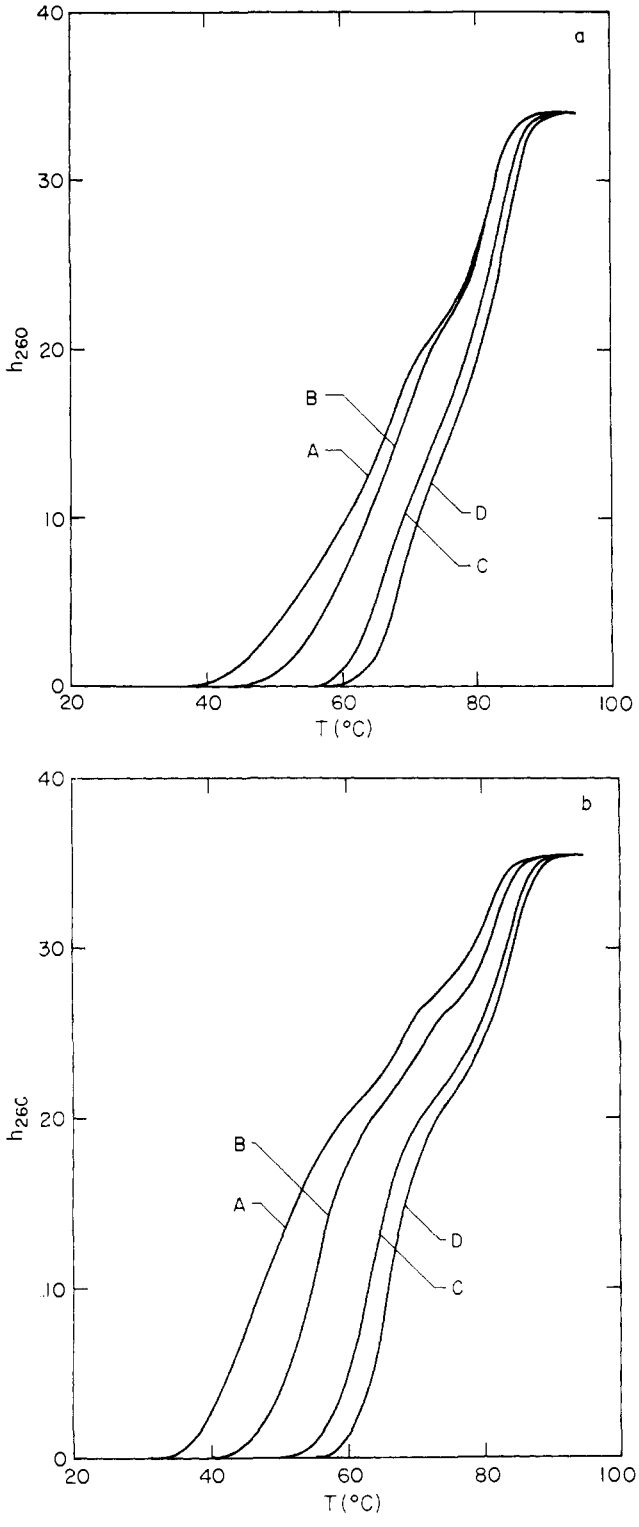


FIGURE 3: Melting profiles of nucleohistones in various buffers A, B, C, and D with varied ionic strengths (see Materials and Methods). (a) Control, (b) 1.0 M NaCl-treated nucleohistone, and (c) 3.0 M NaCl-treated nucleohistone.

involved in these cases (melting band I for native nucleohistone and melting bands III and IV for 3.0 M NaCl-treated nucleohistone). Nucleohistone treated by NaCl from 0.35 to 2.0 M show three parallel shifts as in Figure 4b.

Table II summarizes the effects of ionic strength on the three melting bands. This effect is largest for melting band I, intermediate for III and smallest for IV. Since charge neutralization of the phosphate lattice of DNA is the main factor determining the ionic strength effect on melting temperature (Dove and Davidson, 1962), we may conclude that different DNA segments of nucleohistone are electrostatically shielded by histone binding to different extents. Those segments (free DNA) corresponding to melting band I are least shielded. The shielding effect is intermediate for those segments of DNA belonging to melting band III and greatest for those belonging to melting band IV.

The $T_m$ values for each of the three melting bands are plotted in Figure 5 as a function of $pNa^+$ ($-\log Na^+$). These yield straight lines of different slopes. As shown in Figure 5, the $T_m$ values for pure pea bud DNA fall on the line for melting band I, in agreement with the interpretation that melting band I is due to free DNA segments of nucleohistone. Melting bands III and IV, both stabilized by histone, are due to DNA segments shielded to different extents.

In summary, our results agree with the picture that melting bands III and IV are due to two different extents of electrostatic shielding of DNA phosphates by histone binding. The difference is reduced at higher ionic strength because of stronger coupling effect on DNA melting. At high ionic

TABLE II: Effect of Ionic Strength on Melting Temperature (°).[a]

| Buffers[b] | $\Delta T_{mI}$ | $\Delta T_{mIII}$ | $\Delta T_{mIV}$ |
|---|---|---|---|
| B–A | 8.5 | 3.0 | 0.6 |
| C–A | 18.1 | 10.2 | 2.7 |
| D–A | 22.2 | 11.8 | 3.8 |

[a] Values are averaged from the results of nucleohistones treated by 0, 0.35, 0.60, 1.0, 1.5, 2.0, and 3.0 M NaCl. [b] $(\Delta T_{mi})_{B-A} = (T_{mi})_B - (T_{mi})_A$, where A, B, C, and D are buffers of varied ionic strength (see Materials and Methods).

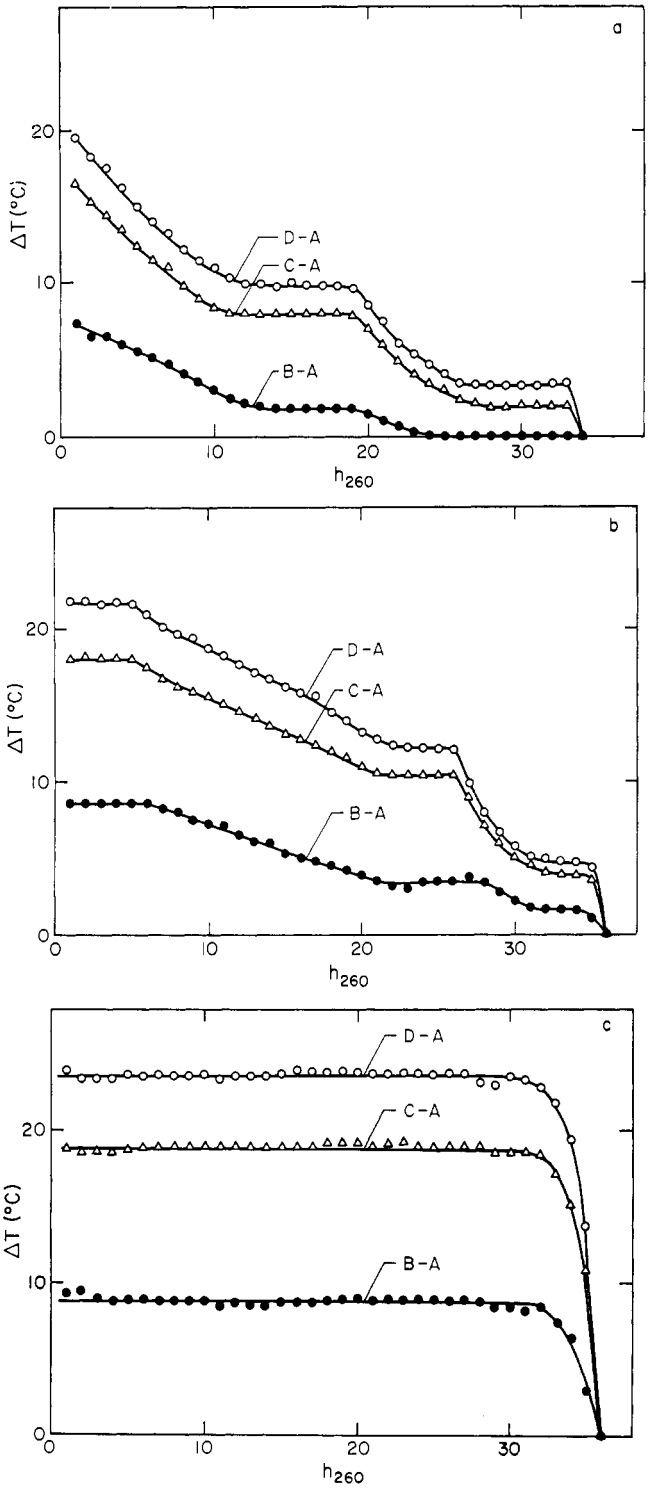


FIGURE 4: Degrees ($\Delta T$) of shift of a melting profile in buffer A to that in other buffers, B, C, and D as a function of the extents of melting. $\Delta T$ are temperature differences from profile A to profiles B, C, or D, respectively, at any given $h_{260}$. They are noted as B–A, C–A, and D–A. (a) Control, (b) 1.0 M NaCl-treated nucleohistone, and (c) 3.0 M NaCl-treated nucleohistone.

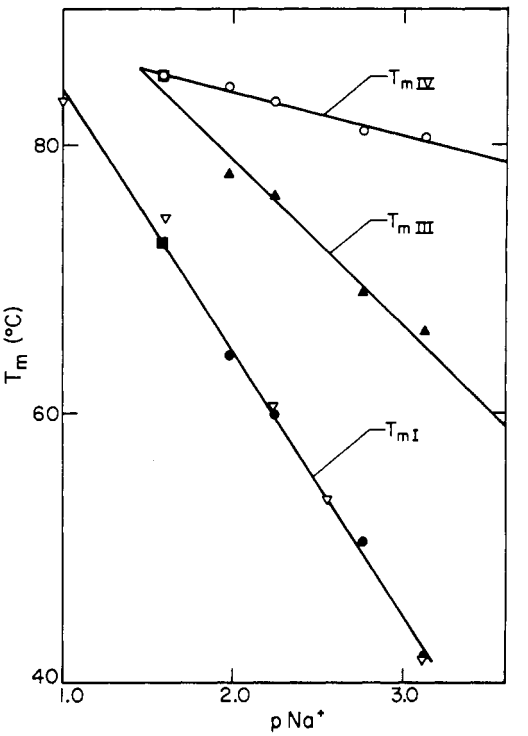


FIGURE 5: Linear dependence of melting temperatures of each melting band on the logarithm of ionic strength ($pNa^+ = -\log Na^+$). ●—●, $T_{mI}$; ▲—▲, $T_{mIII}$; ○—○, $T_{mIV}$; ▽, pea bud DNA; ⊡, melting band a in buffer E; □, melting band b in buffer E (see text).

strength, $2.6 \times 10^{-2}$ M, they are essentially equally stable and melt together. The sources of melting band II exhibit heterogeneous responses toward ionic strength. However, they all melt together and with free DNA segments (melting band I) at high ionic strength.

*Renaturation of Nucleohistone.* It is known that binding of histone to DNA as in nucleohistone causes DNA to renature more rapidly than is the case for pure DNA (Olivera, 1966; Olins, 1969). Since histone binding to DNA causes two different degrees of thermal stabilization of nucleohistone, we ask which is responsible for the renaturation effects. From Figure 2 we know that melting band III extends from ~57 to ~75° with a peak at 66°. Melting band IV extends from ~73 to ~90° with a peak at 81°. Melting between 60 and 70° is completely due to melting band III and that above 75° is completely due to melting band IV. The hyperchromicity between 60 and 70°, $\Delta h_{60-70°}$, which occurs during denaturation after reannealing can be regarded as a measure of DNA segments belonging to melting band III which have renatured. By the same token, hyperchromicity above 75°, $\Delta h_{T>75°}$, which occurs during denaturation after reannealing can also be used as a measure of DNA segments belonging to melting band IV which have been renatured. By these criteria we study the effect of the intactness of one kind of DNA segment on the restoration of the other during renaturation.

Five separate experiments were done by predenaturing nucleohistone to five different temperatures, 55, 65, 73, 80, and 95°, respectively. The five separate results are combined, shown in Figure 6 and summarized in Table III. When nucleohistone is predenatured up to 55°, at which free DNA segments, but not histone-bound DNA regions, are melted, there is complete renaturation. This result agrees with Olin's finding (1969) that nearly all DNA is renatured when nucleoprotein is predenatured to a temperature lower than that of the melting of the histone–DNA complex. Thus, if histone-bound DNA segments are kept intact, the helix conformation of other free DNA segments can be restored even if they were previously denatured.

However, when predenaturation is carried to the level of

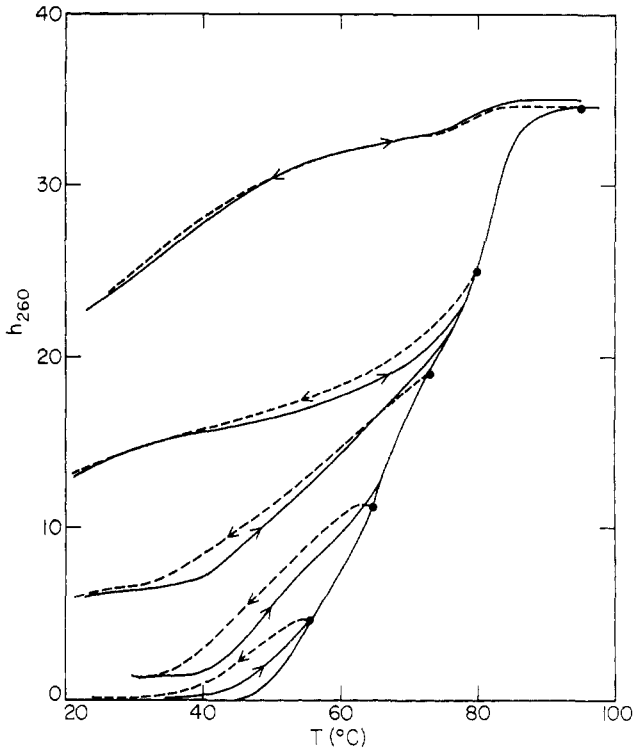


FIGURE 6: Denaturation, renaturation, and redenaturation of native nucleohistone. ● at 55, 65, 73, 80, and 95° are degrees of temperature to which nucleohistones have been separately predenatured. -------, renaturation; ——, redenaturation. They represent the results from these five separate experiments.

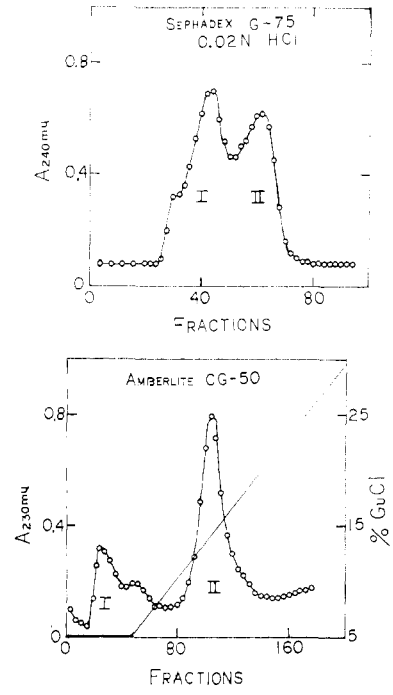


FIGURE 7: Fractionation studies. (a, top) Of CNBr-treated calf thymus histone II by Sephadex G-75 column chromatography. (b, bottom) Of calf thymus histone IIb2-N and IIb2-C by Amberlite CG-50 column chromatography using Gdm·HCl gradient.

melting of histone-bound regions, denaturation is less reversible. In general the amount of renaturation is reduced as more histone-bound regions are predenatured. Table III summarizes the results of renaturation of histone-bound DNA segments when the nucleohistone was predenatured to various temperatures. The melting of histone-bound DNA segments belonging to melting band III has no effect on those of melting band IV. This is shown by the completely native state of melting band IV even when predenaturation has been taken to 73° at which melting band III is nearly completely melted. The most interesting result of Table IV is the close relation between the intactness of the DNA segments of melting band IV and the renaturation of the DNA segments of melting band III. The more extensively IV is denatured, the less III is capable of renaturation.

TABLE III: Renaturation of Melting Band III and the Intactness of Melting Band IV.

| Predenatured to $T$ (°C) | $\Delta h_{60-70°}$ [a] | $\Delta h_{T>75°}$ | $h_{max}$ [b] |
|---|---|---|---|
| Native | 9.6 (100) | 13.8 (100) | 34.5 |
| 55 | 9.6 (100) | 13.8 (100) | 34.8 |
| 65 | 6.8 (71) | 14.0 (100) | 34.5 |
| 73 | 4.4 (46) | 14.3 (100) | 35.5 |
| 80 | 1.9 (20) | 12.6 (91) | 34.0 |
| 95 | 0.8 (8.3) | 1.7 (12.3) | 35.0 |

[a] $\Delta h_{T_i-T_j} = h(T_j) - h(T_i)$ in redenaturation. [b] $h_{max}$ is the total hyperchromicity of the sample after redenaturation. ( ) = % renatured with respect to native nucleohistone.

*Preparation of Histone IIb2-N- and IIb2-C-Half-Molecules.* The distribution of basic amino acids is uneven within histone molecules (Bustin *et al.*, 1969; DeLange *et al.*, 1969; Ogawa *et al.*, 1969; Iwai *et al.*, 1969). In the cases of histones IV and IIb2, the N-terminal half-molecule has *ca.* 0.32 basic amino acid per residue while the C-terminal-half has but *ca.* 0.20. It has already been shown that melting bands III and IV are due to different extents of electrostatic shielding of DNA by histone binding and that the DNA segments of these two melting bands may be physically connected. It would appear that the two melting bands, III and IV, may be due to the characteristically uneven distribution of basic amino acids along the histone molecule. Histone IIb2 (Iwai *et al.*, 1969) provides the ideal model molecule for study of this matter since it possesses two methionine residues in its center (position 58 and 61 along the total of 125 residues). These can be specifically cleaved by CNBr.

Figure 7a shows the fractionation of CNBr-treated calf thymus histone II by Sephadex G-75 column chromatography. CNBr-cleaved histone IIb2 (fraction II) is separated from uncleaved histone IIb1 and IIa (fraction I). Both the N- and C-half molecules of CNBr-cleaved histone IIb2 (fraction II of Figure 7a) are further separated by Amberlite CG-50 column chromatography with a Gdm·HCl gradient (Figure 7b). Fraction I, the run-off peak with 5% Gdm·HCl, appears as a single band in polyacrylamide gel electrophoresis. Fraction II, eluted with 12% Gdm·HCl, appears as two bands in such electrophoresis. It presumably contains both histone IIb2-N-half-molecules and uncleaved histone IIb1 and IIa, since these two fractions (Figure 7a) are not well separated. The histone IIb2-N fraction was further purified by Sephadex C-75 column chromatography. Pure fractions of histone IIb2-N- and IIb2-C-half-molecules have been thus

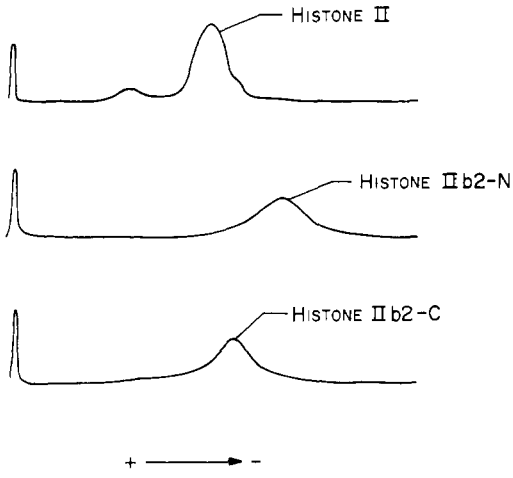


FIGURE 8: Densitometer tracing of polyacrylamide gel disc electrophoresis of calf thymus histone II, IIb2-N, and IIb2-C fractions.

obtained and Figure 8 shows results of polyacrylamide gel electrophoresis of whole histone II, IIb2-N, and IIb2-C fractions. Both histone IIb2-N and IIb2-C fractions have been further analyzed by their amino acid compositions. These are compared to their calculated compositions from the known sequence (Iwai *et al.*, 1969) as shown in Table IV. The fractions thus obtained are therefore pure and are histone IIb2-N- and IIb2-C-half-molecules.

*Reconstitution of Histone–DNA Complexes by Continuous Salt Gradient Dialysis in the Presence of Urea.* The reconstitution of histone II, and of peptides IIb2-N and IIb2-C to DNA was done by the salt gradient procedure (Huang *et al.*, 1964; Olivera, 1966; Olins, 1969) as modified by use of the presence of urea (Bekhor *et al.*, 1969; Huang and Huang, 1969; Shih and Bonner, 1970a). The presence of urea appears to minimize protein–protein interaction and maximize specific complex formation between histone and DNA.

TABLE IV: Amino Acid Compositions of the N-Terminal (-N) and C-Terminal (-C) Fragments of Histone IIb2.[a]

| Amino Acid | Amino Acid Residues/Fragment | | | |
|---|---|---|---|---|
| | Histone IIb2-N | | Histone IIb2-C | |
| | Measured | Calcd[b] | Measured | Calcd[b] |
| Asp | 2.2 | 2 | 4.2 | 4 |
| Thr | 2.0 | 2 | 5.7 | 6 |
| Ser | 6.5 | 7 | 6.7 | 7 |
| Glu | 4.4 | 4 | 6.3 | 6 |
| Pro | 4.8 | 5 | 1.1 | 1 |
| Gly | 3.1 | 3 | 3.3 | 3 |
| Ala | 5.8 | 6 | 7.4 | 7 |
| Cys | 0 | 0 | 0 | 0 |
| Val | 4.7 | 5 | 3.8 | 4 |
| Met | 0 | 0 | 0 | 0 |
| Ile | 1.2 | 1 | 4.1 | 4 |
| Leu | 1.1 | 1 | 5.4 | 5 |
| Tyr | 2.8 | 3 | 2.1 | 2 |
| Phe | 0 | 0 | 2.1 | 2 |
| His | 1.1 | 1 | 2.5 | 2 |
| Lys | 13.7 | 14 | 5.8 | 6 |
| Arg | 3 | 3 | 5.2 | 5 |

[a] Histone IIb2 is specifically cleaved at methionine peptides by CNBr. [b] Calculated from amino acid sequence of histone IIb2 published by Iwai *et al.* (1969).

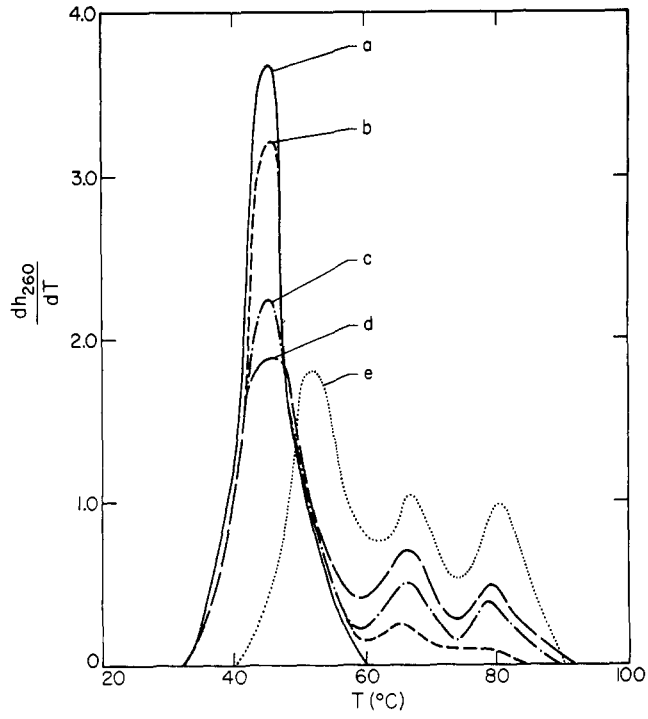


FIGURE 9: Melting spectra of reconstituted histone II-DNA complexes in buffer A. Input ratios +/− (ratio of cation on histones to phosphates on DNA) are 0, 0.12, 0.3, 0.4, and 0.6 for spectra a, b, c, d, and e, respectively.

The data of Figure 9 present the derivative melting profiles of the histone II–DNA complexes in buffer A. When more histone II is complexed to DNA, the free DNA band (45°) diminishes and this is accompanied by an increase of bands at 66 and 80°. It is clear that histone II alone stabilizes DNA to two different degrees (bands at 66 and 80°) just as is true for native nucleohistone (Figure 1). The melting of free DNA segments at higher histone coverage of DNA occurs at temperatures several degrees higher than is characteristic of free DNA molecules as shown in Figure 9e. The reason for this will be discussed below.

Histone IIb2-N- (basic portion) half-molecules when complexed to DNA stabilize the latter to 70°. The results are shown in Figure 10. Histone IIb2-C- (less basic portion) half-molecules stabilize DNA only to 57° (d in Figure 11). At lower coverage of DNA by histone IIb2-C (b and c in Figure 11) the melting bands are asymmetric with a peak corresponding to free DNA band and a shoulder near 57°. These can be resolved into two symmetric bands, one at 45° (free DNA band) and the other at 57° (histone IIb2-C-bound DNA band). It appears that histone IIb2-C-half-molecules with 0.20 basic amino acid/residue stabilize DNA to 57° while histone IIb2-N-half-molecules with 0.32 basic amino acid/residue stabilize DNA to 70°. Both are *ca.* 10° below the 66 and 80° of the two melting bands of histone II-DNA and of the melting bands III and IV of nucleohistone.

If the two half-molecules, IIb2-N and IIb2-C, are mixed

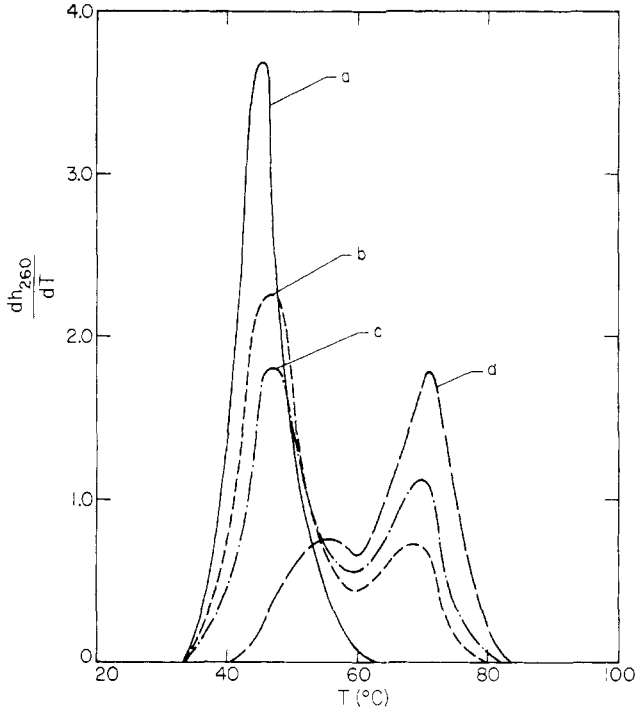


FIGURE 10: Melting spectra of reconstituted histone IIb2-N–DNA complexes in buffer A. Input ratios +/− are 0, 0.4, 0.7, and 1.3 for spectra a, b, c, and d, respectively.

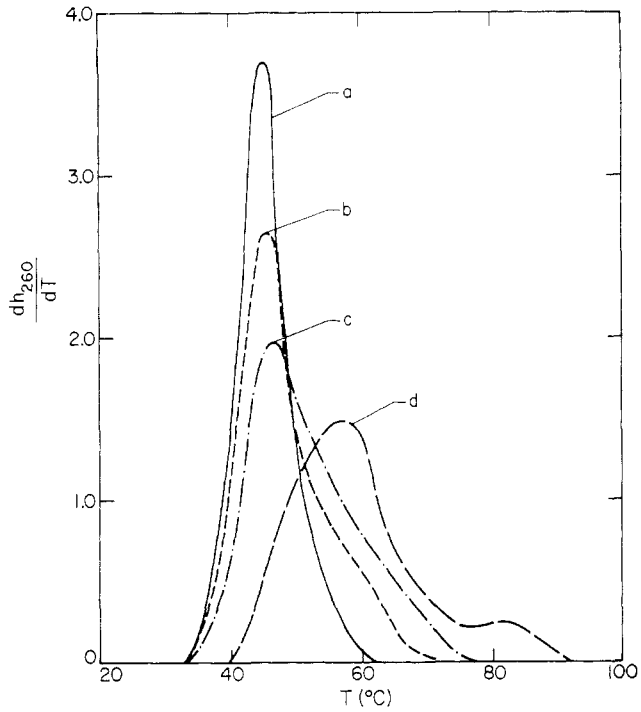


FIGURE 11: Melting spectra of reconstituted histone IIb2-C–DNA complexes in buffer A. Input ratios +/− are 0, 0.4, 0.7, and 1.3 for spectra a, b, c, and d, respectively.

and reconstituted with DNA the derivative melting profile (Figure 12) is broad. It can however be resolved into three bands, at 45, 57, and 70°, as illustrated in Figure 12C. Thus, when the whole histone molecule is broken into two fractions and the mixture reconstituted, they do not form a complex identical with that formed by the intact histone molecule. The two fractions do however appear to stabilize DNA independently.

The data of Figure 13 show that there is a linear dependence of $T_m$ of basic protein–DNA complexes on the log of the basic amino acids per residue. The data are derived from experiments with DNA complexed with four basic proteins, polylysine (or polyarginine), protamine (Shih and Bonner, 1970b), histone IIb2-N and histone IIb2-C. The fraction of basic amino acids per total amino acids is 1.0 for polylysine (or polyarginine), 0.7 for protamine, 0.32 for histone IIb2-N, and 0.20 for histone IIb2-C. It is therefore clear that the extent of thermal stabilization of DNA by a basic protein depends on the effectiveness with which that basic protein electrostatically shields DNA.

## Discussion

*A Model for the Two Melting Bands Caused by Histone Binding.* Binding of histone to DNA results in two different degrees of stabilization. This results in melting bands III at 66° and IV and 81° in buffer A. Selective removal of lysine-rich histone I by 0.60 M NaCl reduces the amplitudes of both melting bands. Thus it is unlikely that either band is due to stabilization of DNA by a particular class of histone. This is also shown by the fact that reconstitution of slightly lysine-rich histone II to DNA yields a nucleohistone which exhibits the two melting bands.

Our model is one in which the two melting bands, III and IV, are due to different degrees of stabilization of DNA by the two different ends of histone molecules. The two ends possess different contents of basic amino acids. A segment of DNA covered by the more basic end of a histone is more shielded electrostatically than is a segment of DNA covered by the less basic end of the histone. The more stabilized segments melt at a higher temperature (melting band IV). The less stabilized segments melt at a lower temperature (melting band III).

This model is supported by the following facts. (1) Histone I (Bustin *et al.*, 1969), histone IIb2 (Iwai *et al.*, 1969), and histone IV (DeLange *et al.*, 1969; Ogawa *et al.*, 1969) all exhibit an uneven distribution of basic amino acids along their length. (2) The fractional areas under melting bands III or IV remain nearly identical as successively more histone is removed (Table I). (3) The effect of solvent ionic strength on melting temperature is largest for melting band I, intermediate for III and smallest for IV. Thus, electrostatic shielding of DNA by protein is smallest for free DNA segments, intermediate for the segments which yield melting band III, and largest for segments which yield melting band IV (Figures 6, 7, 8, and 9, and Table II). (4) The segments of melting bands III and IV are physically coupled as shown by renaturation experiments (Figure 10 and Table IV). (5) Histone IIb2-N-half-molecules stabilize DNA to 70° which is 13° higher than 57°, the melting temperature characteristic of DNA covered by histone IIb2-C-half-molecules. The $T_m$ of melting band IV (81°) is 15° higher than that of melting band III (66°).

According to our model, the length of DNA covered by a single histone molecule may be divided into two segments which melt rather independently of each other and of their neighboring free DNA segments. It may be asked whether this model is physically justified with respect to cooperativity of DNA melting (Dove and Davidson, 1962; Crothers *et al.*, 1965). Cooperativity of melting depends upon ionic strength

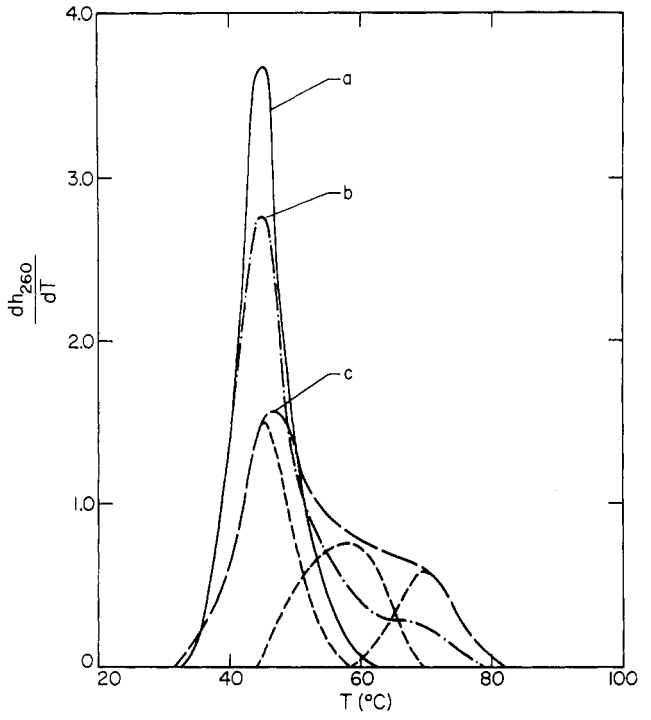


FIGURE 12: Melting spectra of reconstituted histones (IIb2-N + IIb2-C)-DNA complexes in buffer A. Input ratios (+ (N), + (C))/− are (0,0), (0.2, 0.15), (0.4, 0.3) for spectra a, b, and c, respectively. ------, tentative resolutions of melting spectra c into three bands with peaks at 45, 57, and 70°, respectively.

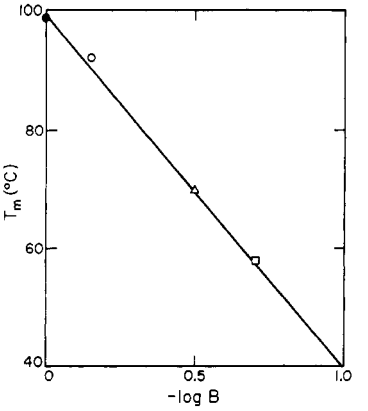


FIGURE 13: Dependence of the melting temperatures in buffer A of basic protein–DNA complexes on their basicity. B is the fraction of basic amino acid per residue of the proteins. (●) Polylysine–DNA, (○) protamine–DNA (Shih and Bonner, 1970b), (△) histone IIb2-N–DNA, and (□) histone IIb2-C–DNA.

(Dove and Davidson, 1962; Huang, 1968; Gruenwedel and Hsu, 1969), base composition (Inman and Baldwin, 1964; Crothers *et al.*, 1965), and even base sequence (Crothers, 1968). The reduction of cooperativity of melting at low ionic strength is due to the larger difference in thermal stabilities of neighboring G-C and A-T pairs. Thus, the binding of a basic protein to DNA, which induces a large difference in thermal stabilities from that of neighboring free DNA regions, will reduce cooperativity of melting, especially at low ionic strength. $T_{mI}$ is 42°, $T_{mIII}$ is 66°, and $T_{mIV}$ is 81°. These differences are so large that coupling of melting among such different segments will be weak. The length of DNA segment which will melt cooperatively due to coupling effects will therefore be much shorter in a nucleoprotein than in pure DNA.

When tetralysine is bound to DNA the complex exhibits only monophasic melting (Olins *et al.*, 1968). Oligolysine (14–18 residues) when bound to DNA exhibits biphasic melting just as does polylysine. Our model of the melting of nucleoprotein proposes that the individual segments of DNA bound to tetralysine molecules are too short to overcome the coupling effects to neighboring free DNA regions but that an oligolysine of 14 residues or more binds to a DNA sequence long enough to introduce an independent melting. Even though the exact configuration of the oligolysine–DNA complex is not known, we can, from the physical length of the molecule, estimate that an oligolysine of 14 residues can bind DNA as short as 7 or as long as 14 nucleotides, depending on whether it binds to one or both strands of DNA. The length of a DNA segment covered by a single basic protein which is required before it can melt as a unit independent of neighboring regions depends on ionic strength. The buffer used by Olins *et al.* (1968) is 0.001 M sodium cacodylate (pH 7.0) which has ionic strength close to that of our buffer A ($Na^+$ ~7.5 × $10^{-4}$ M). Our comparison is therefore meaningful.

The histone IIb2-N-half-molecule contains 57 residues of which 18 are basic. The histone IIb2-C-half-molecule contains 64 residues of which 13 are basic. The DNA coverages of these peptides must be greater than that of oligolysine of 18 or 13 residues because their electrostatic shielding of DNA is less than that of polylysine (Figure 13). It is therefore not surprising to find that the reconstituted complex of histone IIb2-N-DNA and of IIb2-C-DNA exhibit biphasic melting (Figures 11 and 12).

Both the prediction of weakened cooperativity of melting of DNA by binding of basic protein and by media of low ionic strength, and the experimental facts concerning biphasic melting due to oligolysine binding to DNA provide support for our model. It is further supported by the direct evidence that histone IIb2-N- and IIb2-C-half-molecules stabilize DNA to different extents, each with its own melting temperature.

*Effect of Weak Coupling on Melting.* The proposed model separates the basic protein–DNA complex into segments of different thermal stabilities. At low ionic strength the cooperativity of melting is weak and the segments melt rather independently. The coupling of melting, no matter how weak, still exists, however. For example, the complex of tetralysine with DNA does not exhibit biphasic melting but rather monophasic melting with a $T_m$ higher than that of DNA. Because of the weak coupling of melting, a few base pairs at the boundaries of each segment are somewhat different from the base pairs of the interior of the segment.

In the case of free DNA segments, such boundary regions or small gaps between adjacent protein-bound segments will melt at temperatures several degrees higher than does free DNA. As more protein is bound to DNA, the population of such boundaries and gaps is increased and the melting of free DNA segments is correspondingly shifted toward higher temperature. In fact, when histone, or basic polypeptides are bound to DNA, at a cation/anion ratio of *ca.* 0.6, the $T_m$ of free DNA segments is raised by 3–8° (Olins *et al.*, 1967, 1968; Olins, 1969; Shih and Bonner, 1970a,b). Melting band II (52°) of nucleohistones (Figures 1b, 2b, 4b, and 5) may arise partly from the melting of such boundary and gap regions.

Weak coupling effects can of course also influence the

melting of protein-bound DNA segments. In this case, $T_m$ will be lowered by the presence of boundaries. We would expect a reduction of $T_m$ as the size of the protein is decreased. This is true in the case of oligolysine and polylysine binding to DNA (Olins *et al.*, 1968). It is also reflected in the slight increase of $T_m$ of histone II-DNA and histone IIb2-N-DNA complexes as the coverage of DNA by histone is increased (Figures 9 and 10).

The weak melting coupling and its dependence on protein size may explain in part the reduction of $T_m$ of nucleohistones from 66 to 57° or from 80 to 70° when histone IIb2 molecules are cleaved into half-molecules. Thus, when the two ends of a histone molecule are covalently linked as in native molecules, the two segments of DNA bound by these two ends are connected and there is no free DNA gap. In the case of half-molecules, the coupling effect will be less and tend to lower the thermal stability of the complex.

Acknowledgments

We thank our colleagues Professor Norman Davidson and Dr. John Smart for their continuing counsel.

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