Infrared Linear Dichroism Investigations of Deoxyribonucleic Complexes with Histones H2B and H3†

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ABSTRACT: Complexes between DNA and histones H2B and H3 were studied by means of infrared linear dichroism in a wide range of histone to DNA ratios and of different relative humidities. The measurement of the dichroic ratios allows one to determine the secondary structure of DNA in the complexes. It is shown that the progressive addition of histone H2B or H3 to DNA inhibits the structural B → A transition and DNA remains in a B-type form at low relative humidity. A new simple method is proposed to evaluate the amount of A or B forms of DNA when both structures are present. It is found that the B → A transition is fully inhibited when only one molecule of H2B or H3 histone is bound per about three or four turns of DNA helix, respectively. It is proposed that about four to three turns of DNA helix represent the "critical length of DNA" (minimum "cooperative unit") for the B → A transition.

Investigations of DNA–histone complexes have proved to be a useful approach for the understanding of chromatin structure (Van Holde and Isenberg, 1975). In the present investigation, complexes of histones with DNA, histone H3–DNA and histone H2B–DNA, are studied by means of infrared linear dichroism. Different methods have been used for the investigation of gross structure of histone–DNA artificial complexes, such as: x-ray diffraction (Boseley et al., 1976), electron microscopy (Oudet et al., 1975), circular dichroism (Adler et al., 1974; Leffak et al., 1974; Yu et al., 1976a,b), thermal denaturation (Shih and Bonner, 1970; Li and Bonner, 1971; Ansevin and Brown, 1971; Yu et al., 1976a,b). Histone H3 has the amino acid sequence of general histone type, with arginine rich distribution (Bradbury et al., 1972; Delange et al., 1973). Using nuclear magnetic resonance Bradbury showed that the central and C-terminal parts of the H3 sequence have a secondary structure like globular proteins. The basic part of histone H3 is situated in the N-terminal part.

Histone H2B is lysine rich (Delange et al., 1973). The distribution of basic amino acid of the N-terminal part is slightly different than in H3, but the central part of the molecule has an amino acid composition like many globular proteins and is rich in hydrophobic residues.

It has been shown previously that infrared linear dichroism is a very powerful technique to obtain direct results about the secondary structure of DNA (Pilet and Brahms, 1972, 1973; Brahms et al., 1973). We have studied in an earlier investigation the influence of poly(L-lysine) and poly(L-arginine) on the conformation of DNA (Liquier et al., 1975). These complexes are often considered as a simple model to approach histone–DNA interactions. This work presents the study of the conformational B → A transition of DNA in H2B–DNA and H3–DNA complexes as a function of relative humidity. It is shown that in contrast to DNA complexes with poly(L-lysine) and poly(L-arginine), histones H2B and H3 are influencing strongly the B → A structural transition of DNA, and are inhibiting this conformational change. The interpretation of obtained results on H2B and H3 binding to DNA allows one to estimate the "critical length" of DNA (or a "minimum cooperative unit") for this B → A transition which is of about three–four turns of helix.

Materials and Methods

Complex Preparation. Histone fractions H2B and H3 were prepared following method 2 of Johns (1964).

Salmon sperm DNA was purchased from Worthington Chemical Co. and dissolved in 2 M NaCl solutions. The concentrations were determined by UV (ultraviolet) absorbance measurements assuming $\epsilon_{260nm}^{1mg} = 20$ for a 1-cm path cell.

The physical state of the complex depends on the salt concentration and the conditions of mixing (Huang et al., 1974; Bekhor et al., 1969; Li and Bonner, 1971; Leffak et al., 1974; Boseley et al., 1976). DNA–histone complexes begin to form below 0.4 M NaCl.

Histone fractions at moderate and high salt concentrations aggregate (Diggle and Peacocke, 1971; Van Holde and Isenberg, 1975; Sperling and Bustin, 1975).

An increase in ionic strength after a first fast process of rearrangement is followed by a slow aggregation, very sensitive to temperature conditions, and which can effectively be stopped for very long periods of time at 2 °C.

Histone H2B aggregates less than H3 by increasing the ionic strength of the solution. Sperling observed, by electron microscopy, bent rods formed by aggregates of H3 and of H2B (see also Edwards and Shooter, 1969; Boublik et al., 1971; D'Anna and Isenberg, 1972; Sperling and Bustin, 1976). To avoid the aggregation of histones and the too rapid binding of histones to DNA followed by precipitation of the complex, we have used the procedure described below: histone H3 was dissolved in 10^{-3} M NaCl–10^{-3} M DTT (dithiothreitol is necessary to avoid the aggregation of histone H3 at high salt concentrations). Histone H2B was dissolved in 2 M NaCl–5 M urea. The histone solution was then carefully added to the 2 M NaCl–DNA solution to obtain histone/DNA weight ratios between 0.1:1 and 0.6:1. The H2B–DNA mixtures were dialyzed in the cold room ($T \sim 4$ °C) against successive NaCl–urea solutions (5 M urea–2 M NaCl, 5 M urea–0.9 M NaCl, 5 M urea–0.6 M NaCl, 5 M urea–0.3 M NaCl, 5 M urea–0.15 M NaCl, 0.15 M NaCl). The final dialysis solution (e.g., 1–10 mM NaCl) was such as to obtain a NaCl:DNA weight ratio of 5–6% in the sample. The H3–DNA mixtures were directly dialyzed against the appropriate final solution.

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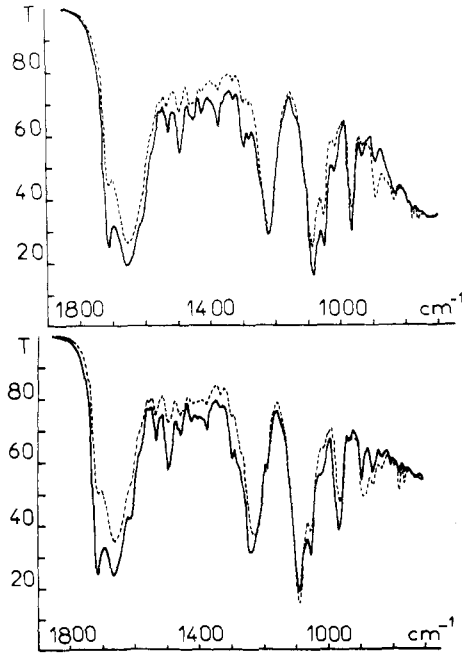

FIGURE 1: (Top) Infrared spectrum of H2B-DNA 0.1:1 complex, high humidity: (—) electric vector of polarized light perpendicular to the orientation axis; (···) electric vector of polarized light parallel to the orientation axis. (Bottom) Same sample, low relative humidity.

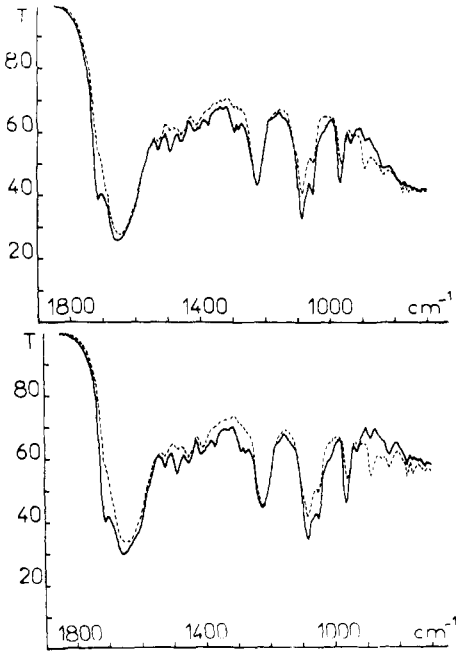

FIGURE 2: (Top) Infrared spectrum of H2B-DNA 0.5:1 complex, high humidity, same notations as Figure 1. (Bottom) Same sample, low relative humidity.

The complexes thus prepared are homogeneous gel-like samples.

The histone:DNA ratios were controlled by infrared spectroscopy using amide I band absorption for the histone and the 1710-cm^{-1} characteristic absorption for the DNA. Histone-DNA weight ratios were r = 0.08:1, 0.20:1, 0.31:1, 0.4:1, and 0.44:1 for H3 and 0.1:1, 0.2:1, 0.24:1, 0.28:1, 0.35:1, and 0.5:1 for H2B.

Melting Curves. Melting profiles of histone-DNA complexes were obtained with a Cary 17 spectrophotometer and also as the derivative plots of the absorbancy vs. temperature. The formation of the H2B-DNA and H3-DNA complexes was followed by comparing their melting points to that of pure DNA under identical salt conditions (5×10^{-4} M NaCl). As in the previous studies of Shih and Bonner (1970), Ansevin and Brown (1971), and Li and Bonner (1971) we are also observing the biphasic melting curves with a lower T_M at 61 °C and a higher T_M at 74 °C for H3 and 87 °C for H2B.

Infrared Measurements. The infrared spectra were recorded on a Perkin-Elmer Model 180 double beam ratio recording spectrophotometer (between 4000 and 700 cm^{-1}) equipped with a wire grid polarizer with KRS 5 support placed in the common beam. The infrared dichroic ratios, $R(\perp/\parallel) = A_\perp/A_\parallel$, were determined from measurements of the spectra of oriented films with the electric field of the incident light parallel and perpendicular to the orientation axis. Samples were oriented at 45° with respect to the slit, thus eliminating the polarization due to the gratings of the spectrophotometer. The relative humidity of the film was obtained and controlled as previously described by measurement of the H_2O absorption band at 3400 cm^{-1}. The dichroic ratio allows one to calculate the angle θ which the transition moment forms with the DNA axis:

$$R(\perp/\parallel) = \frac{\sin^2\theta + g}{2\cos^2\theta + g}$$

where g is the parameter which characterizes the semicrystalline state of the oriented sample and is evaluated by using the value of $R(\perp/\parallel)$ at 1710 cm^{-1} (Pilet and Brahms, 1972; Brahms et al., 1973).

Results

Structural Transitions of DNA Complexes. Figure 1, top, shows the polarized spectrum of the H2B-DNA complex of 0.1:1 weight ratio, recorded with the electric vector of the light parallel and perpendicular to the orientation axis, at high relative humidity (RH 100%). One can observe the following characteristic features of a B family form of DNA: (1) a strongly perpendicular absorption at 1710 cm^{-1}, assigned to the C=O and C=N double bond stretching vibrations, characteristic of the base pairing (Tsuboi, 1970); (2) a nondichroic band at 1230 cm^{-1}, assigned to the antisymmetric stretching vibrations of the phosphate group ν_{as}(OPO); (3) a strongly perpendicular band at 1090 cm^{-1} assigned to the symmetric stretching vibration of the phosphate group ν_s(OPO) (Shimanouchi et al., 1964).

Figure 1, bottom, shows the spectrum of the same complex but at lower relative humidity (RH 66%). One can easily observe that the 1710-cm^{-1} band remains strongly perpendicular, whereas the 1230-cm^{-1} absorption band from nondichroic becomes perpendicular, and the 1090-cm^{-1} band from perpendicular becomes parallel; this spectrum is similar to that obtained for films at low RH conditions and which corresponds to the A form of DNA.

Figure 2, top, shows the spectrum of an H2B-DNA complex with a higher histone to DNA ratio (H2B-DNA of 0.5:1 weight ratio, at high relative humidity). This spectrum is very similar to that of Figure 1, top, i.e. DNA presents a B family form. In contrast, in the spectrum of Figure 2, bottom, which corresponds to the same sample but at low relative humidity, the DNA has remained in a B family form.

In the DNA-H2B complex, at a low protein to DNA ratio (0.1:1 weight ratio), the presence of histone slightly modifies the infrared spectrum. The conformational transitions are still observed when the relative humidity of the film is decreased. One observes that for a histone-DNA ratio of 0.5:1, the B

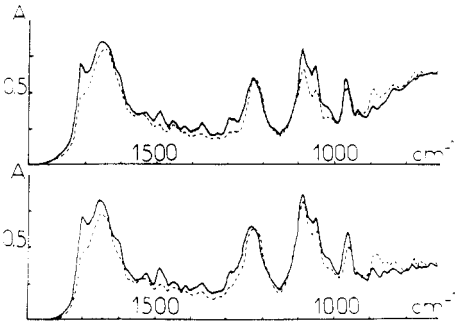

FIGURE 3: (Top) Infrared spectrum of H3-DNA 0.2:1 complex, high humidity, same notations as Figure 1. (Bottom) Same sample, low relative humidity.

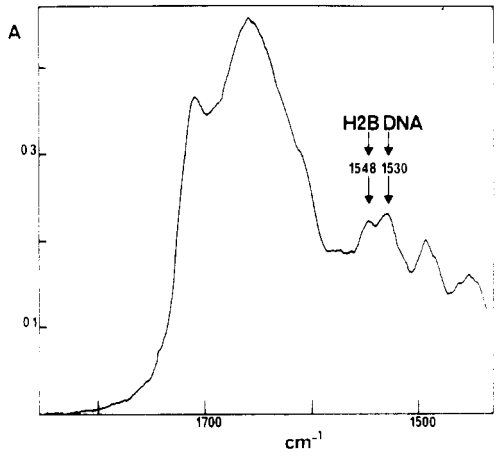

FIGURE 4: Infrared spectrum of H2B-DNA 0.5:1 amide II region.

family form (B*) spectrum remains at low humidity (the 1230-cm^{-1} absorption band is always perpendicularly polarized). At lower relative humidity, the B family form is changed; all dichroic ratios are becoming close to unity and the DNA is completely disoriented.

Similarly the addition of histone H3 prevents the appearance of the A form of the spectrum and inhibits the B → A transition of DNA with decreasing RH, depending on the histone:DNA ratio (Figure 3). Under the same salt conditions, free DNA exhibits a steep B → A conformational transition at about 76% RH.

The region of 800-900 cm^{-1} is very interesting and useful for the qualitative characterization of various forms of DNA by infrared spectroscopy (Brahms et al., 1974) and by Raman spectroscopy (Brown and Petitcolas, 1975; Erfurth et al., 1975). In infrared spectroscopy the A form of DNA at low RH exhibits a parallel band at 860 cm^{-1}, while the B form at high RH yields a parallel band at 830 cm^{-1}. In Raman spectroscopy the A form is characterized by the presence of a band at 807 cm^{-1}. Despite the fact that the definite assignment of these bands is difficult, one considers that the vibrations of the OPO bonds of the phosphodiester chain are generating these absorption bands.

Protein Bands. The thickness of the samples used allows one to observe the amide II absorption of the histone only for high protein:DNA ratios, above 0.35:1. The absorption band of protein is at about 1548 cm^{-1} (Figure 4).

Structural Parameters. We have calculated the orientation of the O⁻⁻P⁻⁻O⁻ bisector angle of the phosphate group with respect to the axis of the DNA molecule using the dichroic ratio of the 1090-cm^{-1} absorption band (see Materials and Methods). The values of θ_{1090} for the high relative humidity B form

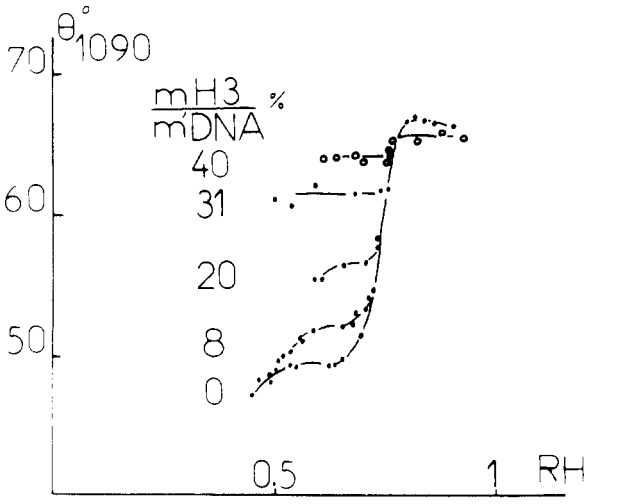

FIGURE 5: H3-DNA; plots of θ_{1090} parameter vs. normalized relative humidity and histone concentration (transition curves).

for the different complexes of histone-DNA are between 66 and 68°, with respect to the DNA axis (in free salmon sperm DNA θ_{1090} = 67°). These values are within experimental error. (If we take into account the maximum errors due to the measurements of the orientation factor and of the phosphate band, these angles are determined with a precision of ±1.5°.) The same calculation for all the values of relative humidity and for each histone/DNA ratio yields plots similar to the one represented in Figure 5 corresponding to the H3-DNA complexes studied. One can observe on this plot at high relative humidities (RH >86%) a first plateau corresponding to a B family form of the DNA, followed by a steep transition and a second plateau for relative humidities between 86 and 58%. If RH is still decreased, disorientation of DNA occurs. The abscissa scale is a relative humidity scale normalized to take into account the different thicknesses of the samples.

The same diagram is obtained with the different H2B-DNA samples as in Figure 5. Similarly one may observe a high relative hygrometry plateau with θ_{1090} about 67°, followed by steep transitions and a low hygrometry plateau. However, with increasing histone to DNA ratios, the second plateau of θ_{1090} corresponding to lower hygrometries is shifted toward the high hygrometry plateau. For a histone-DNA ratio of 0.5:1 both plateaus are occurring at the same level, characteristic of a B form, i.e. θ = 67°. The B → A transition does not exist any more (see Figure 5).

For pure DNA (salmon sperm), the low relative humidity plateau corresponds to that of the A form, and the value of θ_{1090} is of about 49°. Table I shows the values of θ_{1090} calculated for the different samples. These values are between $\theta \sim 49°$ for the A form of free DNA and $\theta \simeq 67$-68° for the B form of free DNA. The genuine value of θ_{1090} for the A form is calculated using an orientation factor, g, slightly different, corresponding to a θ_{1710} value of 80° (A form) instead of 90° (B form). The calculated values for θ_{1090} differ by about 1° for the two extreme values of the orientation factor g.

Discussion

Nature of Conformational Changes. We have previously observed that the formation of complexes with basic homopolypeptides does not change the structural transitions of DNA at different relative humidities (Liquier et al., 1975). In contrast, one of the important results of the present investigation is the suppression of the B → A transitions in the H2B-DNA

TABLE I: Calculated Values of the Phosphate Group Orientation in DNA-H2B and DNA-H3 Complexes.

Form	Sample	m(hist)/ m(DNA) (%)	Residue/ nucleotide	θ_{1090}[b]	% B form
Low humidity 58% < RH < 76%	DNA[a]	0	0	49	
	DNA-H2B	10	0.27	51	9
		20	0.55	54.5	29
		24	0.66	55	31
		28	0.77	57	43
		35	0.96	61	66
		50	1.40	66	94
	DNA-H3	8	0.22	53	20
		20	0.55	57	43
		31	0.85	62	71
		40	1.10	64	83
		44	1.20	64	83
High humidity RH > 86%	DNA	0		67	100
	DNA-H2B	10	0.27	66	100
		20	0.55	67	100
		24	0.66	64	100
		28	0.77	68	100
		35	0.96	67	100
		50	1.40	66	100
	DNA-H3	8	0.22	67	100
		20	0.55	67	100
		31	0.85	66	100
		40	1.10	66	100
		44	1.20	66	100

[a] Salmon sperm DNA. [b] O-P-O bisector angle with respect to DNA axis.

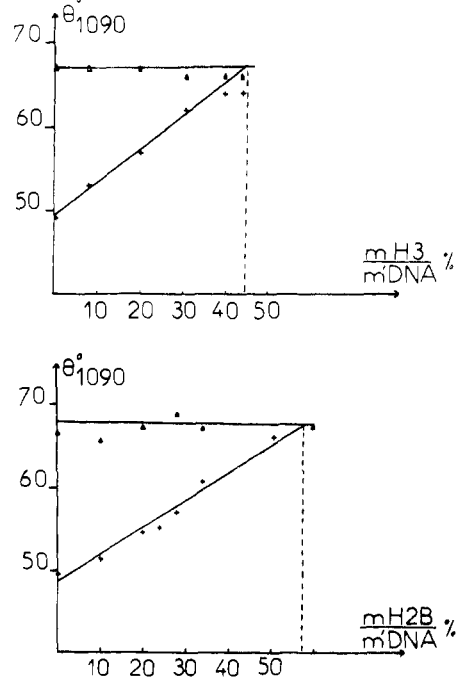

FIGURE 6: (Top) Plot of the θ_{1090} values of the H3-DNA complexes against histone/DNA weight ratios: (+) low humidity plateaus; (Δ) high humidity plateaus. (Bottom) Same figure for H2B-DNA complexes.

and H3-DNA complexes starting with a certain amount of histone content. The same inhibition of the A DNA form at low humidity is also reported for DNA-protamine complexes (Herskovits and Brahms, 1976). (The absence of transition is not due to a lack of variation in relative humidity of the film since it can be controlled by the measurement of the water absorption at 3400 cm^{-1}.)

The primary structures of the histone fractions which inhibit this B → A transition are well known. H2B and H3 are characterized by a dissymmetric distribution of charges and of basic residues along the polypeptide chain (see Van Holde and Isenberg, 1975). The histone H3 has a net positive charge of +18 for the 1-53 first residues of the N terminal and a slight positive charge of +4 for the C-terminal part (Delange et al., 1973). Histone H2B presents a cluster near the N terminal (1-50) of net charge +16 and again a basic part at the C terminal (net charge +5) (Iwai et al., 1972). The basic part of the histones, that is here mainly the N terminal, is known to bind to the DNA phosphate groups during complexation (Boublik et al., 1971; Philips, 1971). The presence of the basic residues on DNA does not change the DNA conformation and it can be therefore concluded that the hydrophobic residues of the histones are responsible for the inhibition of the conformational changes of DNA. This conclusion is compatible with the results of Adler et al. (1974) who found that the N terminal of H2B does not yield the typical H2B-DNA CD spectrum.

Infrared dichroism can readily differentiate between B and C forms of DNA. In the C conformation, the 1230-cm^{-1} absorption band acquires a parallel dichroism which indicates a change of the orientation of the transition moment directed following the O(2)-O(3) line of the phosphate group (Brahms et al., 1973). For all H2B-DNA and H3-DNA samples studied no such parallel band at 1230 cm^{-1} was seen; we have observed at high and low relative humidity the parameters of a B family form.

One may propose that the low humidity plateau of θ_{1090} = f(RH) plots is due to the coexistence of the two A and B forms in various relative amounts rather than to the appearance of a new DNA conformation. In fact, the measured frequencies of the infrared bands at 860 and 830 cm^{-1} of the complexes are always those of A or B form DNA, respectively. Furthermore, the values of θ_{1090} for this DNA remain between the two limits of θ_{1090}, 49° for an A form and 67° for a B form (see Table I). Figure 6 shows that θ_{1090} is a linear function of the ratio $r = m(\text{histone})/m'(\text{DNA})$, where m and m' represent weight fraction of histone and DNA, respectively. We call r_l (for r_{limit}) the value of r corresponding to the angle $\theta_{1090} = 67° = \theta_B$. Infrared measurements allow us to estimate accurately the percentage of A and B forms for each histone-DNA complex. The percentage of B form, k, is given by the formula $k = (\theta_{1090} - \theta_A)/(\theta_B - \theta_A)100$. Figure 6 can thus be interpreted by considering the amount of DNA remaining in the B form at low relative humidity as proportional to the amount of histone in the complex $((r/r_l)100)$.

One must thus emphasize that infrared spectroscopy enables us not only to recognize the various forms of DNA, but is successful in measuring quantitatively the percentage of DNA in one or the other conformation in a mixture of these two forms. This is not possible to recognize by x-ray diffraction.

The Notion of "Critical Length" of DNA for Conformational B → A Changes. Figures 5 and 6 clearly indicate the fundamental character of B → A transition and allow one to make the following observations and conclusions. (1) The B → A transiton of DNA is highly cooperative as shown in Figure 5 and the presence of relatively small amounts of histones

significantly affects this process. (2) The B → A transition of DNA is fully inhibited when 1.3 or 1.7 amino acid residues of H3 or of H2B histones, respectively, are bound per nucleotide residue of DNA. It is known that the N-terminal part of these histones is the binding side (see above and the introductory statement). One phosphate residue of DNA is bound to one basic amino acid residue (Latt and Sober, 1967a,b). Thus, the 18 or 16 adjacent phosphates residues correspond to the minimum length of DNA to which one H3 or H2B molecule is bound by its N-terminal part. A segment of DNA of 1000 nucleotides or 500 base pairs long will be in a frozen B form when 170 or 210 positively charged amino acid residues of H3 or of H2B, respectively, are bound to its phosphate groups. As a result this entire region of DNA will lose its conformation flexibility. Under the conditions used in the present study, the method of preparation of histone-DNA complexes by progressive dialysis from high to low ionic strength (~1 mM NaCl) ensures the condition of *equilibrium* distribution of bound histones.[1] This assumption is supported by the *linear* dependence of the angle θ_{1090} vs. the histone/DNA ratio for different complexes shown in Figure 5. One may thus visualize such a DNA region of 500 base pairs long with more or less equidistantly distributed histone molecules. With these assumptions a simple calculation leads to determination of the "critical length" of DNA between two neighboring N-terminal bound histone H3 or H2B molecules as equal to about *4.3 to 3 turns of helix*. This represents the "critical length" of DNA for conformational change B → A in the presence of complexes with histones. This estimate of the "critical length" of DNA corresponds to a "minimum cooperative unit" for the B → A transition and can be compared with another structural transition of DNA, the B → C transition, which is, in contrast, of gradual character (Brahms et al., 1973). In fact, the enthalpy of the B → C transition was determined recently and is of about ΔH° = 10 kcal, which corresponds to a very small "cooperative unit" of one or a few base pairs only (Brahms et al., 1976). It is difficult to compare this "cooperative unit" for the B → A transition with the "cooperative segment" for helix → coil transitions ("melting") since for the latter the data are not yet clearly determined.

It is tempting to consider that the notion of "critical length" of free DNA may have important biological implications. Such biological processes for which a great conformational flexibility is required will be rather inhibited by the binding of histone or alternatively one will have to consider other mechanisms.

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[1] This is also in agreement with the studies of Burton et al. (1975) who showed that during the dialysis from 2 M NaCl to lower salt concentrations DNA and histones are in thermodynamic equilibrium. At low ionic strength, under the conditions used in the present studies, i.e. up to 10 mM NaCl, the binding of histone to DNA is essentially a noncooperative process, as demonstrated convincingly by Renz and Day (1976).