base further experimentation in this field.

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The Role of the Arginine-Rich Histones in the Maintenance of DNA Conformation in Chromatin†

Vaughn Vandegrift,‡ Martin Serra, Dexter S. Moore,§ and Thomas E. Wagner*

ABSTRACT: The selective extraction of all histone proteins, except the arginine-rich histones III and IV, from calf thymus chromatin, is accomplished by a procedure which has been shown not to allow the redistribution of histones between different sites along the DNA during extraction. The resulting arginine-rich nucleohistone has been compared to native chromatin and a reconstituted Ca^{2+} nucleohistone IV complex using circular dichroism spectroscopy. In the presence of 2.5 mM Ca^{2+} these three preparations display almost identical DNA circular dichroism spectra. The role of histone IV and Ca^{2+} in the maintenance of DNA conformation in chromatin is discussed. Theoretical calculations are presented which interpret the circular dichroism of chromatin and arginine-rich nucleohistone in terms of a superhelical tertiary structure for the DNA component of these systems.

Chromatin, the interphase chromosomal material from eukaryote cells, is a complex macromolecular association of DNA and protein. The proteins associated with this chromatin complex may be divided into two groups: non-histone chromosomal proteins and histone chromosomal proteins. Although evidence has been presented for the involvement of both histone (see Wilhelm *et al.,* 1971) and non-histone (see Spelsberg *et al.,* 1972) chromosomal proteins in template restriction, the precise role of these chromosomal protein classes in genetic regulation remains uncertain. It is clear, however, that the histone chromosomal proteins are, in some way, responsible for the structural integrity of chromatin. The fiber X-ray diffraction pattern (Garrett, 1968; Richards and Pardon, 1970), circular dichroism spectrum (Shih and Fasman, 1970; Simpson and Sober, 1970; Wilhelm *et al.,* 1970; Henson and Walker, 1971; Permogorov *et al.,* 1970; Wagner and Spelsberg, 1971), hydrodynamic

†From the Department of Chemistry, Ohio University, Athens, Ohio 45701. *Received April 11, 1974.* We thank the American Cancer Society (Grant NP-123) and the Research Corporation for their generous support of this work.

‡Present address: Department of Chemistry, Illinois State University, Normal, Illinois 61761.
§Present address: Department of Chemistry and Chemical Biodynamics Lab., University of California, Berkeley, California 94720.

properties (Sponar *et al.*, 1970; Henson and Walker, 1970; Wilhelm *et al.*, 1970; Toczko *et al.*, 1972), thermal denaturation profiles (Li and Bonner, 1971), and microscopic organization (Allfrey *et al.*, 1963; Mirsky *et al.*, 1968) of chromatin are all markedly altered upon the selective removal of the histones.

Since chromatin contains so many macromolecular components in its composition it is impossible at present to address the problem of the "total structure" of chromatin. Because of the genetic importance and the well-understood primary and secondary structure of DNA, the central organizing macromolecule of chromatin, most discussion of chromatin structure has focused upon the conformation of DNA in chromatin. Both X-ray diffraction (Pardon *et al.*, 1967; Pardon and Wilkins, 1972) and optical activity spectroscopy (Tuan and Bonner, 1969; Shih and Fasman, 1970) clearly indicate that the geometry of the DNA molecule in chromatin is markedly different from the geometry of purified DNA. One of the major experimental gambits in chromatin structural studies during the past several years has been to attempt to determine which chromosomal proteins are responsible for maintaining the unique DNA conformation found in chromatin. Since all structural changes in chromatin observed to date have been associated with the removal of the histone class of chromosomal proteins it has been assumed that one or more of the histone proteins has this structural role in chromatin. Present research is focused upon determining which histone or histones is (are) involved in maintenance of DNA conformation in chromatin. Using the selective salt extraction procedures of Ohlenbusch *et al.* (1967) the five histone fractions have been sequentially removed from chromatin and the effects of their removal on DNA conformation monitored using X-ray diffraction (Richards and Pardon, 1970; Garrett, 1968; Skidmore *et al.*, 1973), circular dichroism spectroscopy (Simpson and Sober, 1970; Wilhelm *et al.*, 1970; Henson and Walker, 1971; Wagner and Spelsberg, 1971), and hydrodynamic measurements (Henson and Walker, 1970; Fredericq and Houssier, 1967; Wilhelm *et al.*, 1970). Although a sharp transition from native chromatin DNA to that of free DNA is observed in each of these studies a controversy remains concerning which histone(s) dissociate(s) from chromatin concomitant with this transition. Simpson and Sober (1970), Henson and Walker (1971), and Wilhelm *et al.* (1970) using circular dichroism spectroscopy, Garrett (1968) using X-ray diffraction, and Henson and Walker (1971) using hydrodynamic measurements all observed the DNA transition in chromatin to take place upon the removal of the moderately lysine-rich histones (IIb_2 and IIb_1). Wagner and Spelsberg (1971) using circular dichroism spectroscopy, Richards and Pardon (1970) and Skidmore *et al.* (1973) using X-ray diffraction, and Fredericq and Houssier (1967) and Wilhelm *et al.* (1970) using hydrodynamic measurements observed the release of histone constraint upon chromatin DNA concomitant with the selective removal of histone IV. One possible explanation of these contradictory results is suggested by the work of Clark and Felsenfeld (1971) who were able to show that about 40% of the proteins in chromatin were exchanged at the relatively low salt concentration of 0.6 M NaCl. This redistribution of histones along DNA during the salt extraction of selected histones, of course, considerably clouds the significance of the results of these salt extraction studies.

In our present work we have selectively extracted all histone proteins except the arginine-rich histone (III and IV) from chromatin using low salt and tRNA (Ilyin *et al.* 1971), a procedure shown not to allow the redistribution of histones between different sites along the DNA (Ilyin *et al.* 1971; Varshavsky *et al.*, 1972). The resulting arginine-rich nucleohistone has been compared to native chromatin and a reconstituted Ca^{2+} nucleohistone IV complex (Wagner and Vandegrift, 1972) in the presence of 2.5 mM Ca^{2+} using circular dichroism spectroscopy and was found to display a DNA circular dichroism essentially identical with these two systems. The role of histone IV and Ca^{2+} in the maintenance of DNA conformation is discussed. Theoretical calculations are presented which interpret the circular dichroism spectra of chromatin and arginine-rich nucleohistone in terms of a superhelical tertiary structure for the DNA component.

Materials and Methods

Chromatin Preparation: Chromatin was prepared from calf thymus tissue by a slight modification of the method of Panyim *et al.* (1971). Freshly excised calf thymus glands were frozen on Dry Ice and stored at Dry Ice temperature until use. Frozen tissue was fragmented into small pieces, soaked briefly in 20 ml. of grinding medium (0.25 M sucrose–0.01 M $MgCl_2$–0.01 M Tris (pH 8.0)–0.05 M $NaHSO_3$) per gram of tissue, and then homogenized for 3 min at 30 V and then 1 min at 60 V in a Waring Blender. The homogenized tissue was then filtered through four layers of cheesecloth followed by first two and then four layers of Miracloth. The filtrate was centrifuged at 480*g* for 10 min. The resulting supernatant was discarded and the pellet resuspended in grinding medium and again centrifuged at 480*g* for 10 min. The pellet was washed repeatedly in this fashion until the supernatant became clear. The resulting nuclear pellet was then homogenized gently for 1 min at 20 V in an EDTA solution (0.025 M EDTA–0.05 M $NaHSO_3$–0.01 M Tris (pH 8.0)) and centrifuged at 4300*g* for 10 min, followed by one repeated homogenization and centrifugation. The resulting thick nuclear pellet was then homogenized in 50 volumes of distilled, deionized water using a hand-driven Teflon homogenizer and centrifuged at 12,000*g* for 10 min. After several homogenizations with distilled water all nuclei are lysed. The resulting crude chromatin was dialyzed overnight against 2 l. of 0.005 M Tris (pH 8.0) to ensure complete hydration. Purified chromatin was prepared from this crude chromatin by sedimentation through 1.7 M sucrose at 60,000*g* for 2 hr. The purified chromatin pellets were dialyzed against the appropriate buffer solutions and then solubilized by shearing in a Virtis homogenizer (30 V for 1.5 min) and clarified by centrifugation at 12,000*g* for 20 min (Bartley and Chalkley, 1973). All of the above procedures were performed at 4°.

Preparation of Arginine-Rich Nucleohistone. Purified solubilized chromatin was incubated with purified whole yeast tRNA (Miles Laboratories) according to the methods of Ilyin *et al.* (1971). Briefly, chromatin (1 mg/ml) was incubated with a 15-fold weight excess of tRNA for 24 hr in 0.001 M EDTA–0.005 M Tris (pH 8.0). The incubation mixture was separated into a depleted chromatin fraction and a tRNA–histone fraction by gel filtration chromatography on a 1.5 × 90 cm Sepharose 4B column (see Figure 1). The resulting partially histone depleted chromatin prepared in this manner was used for circular dichroism studies in the presence and absence of Ca^{2+} and for histone content measurements. For studies in the presence of calcium ion the depleted chromatin as well as native chromatin were

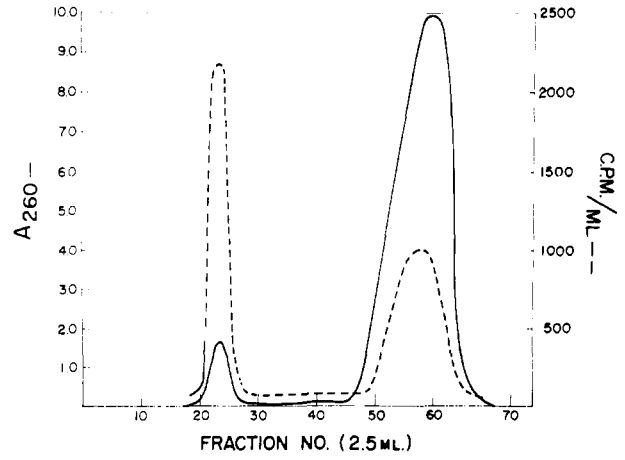

FIGURE 1: Elution profile of tRNA treated chromatin chromatographed on Sepharose 4B. The column (1.5 cm × 90 cm) was equilibrated with 0.005 M Tris (pH 8.0) and was monitored by measuring absorbance at 260 nm and by counts per minute of protein incorporated ^{14}C.

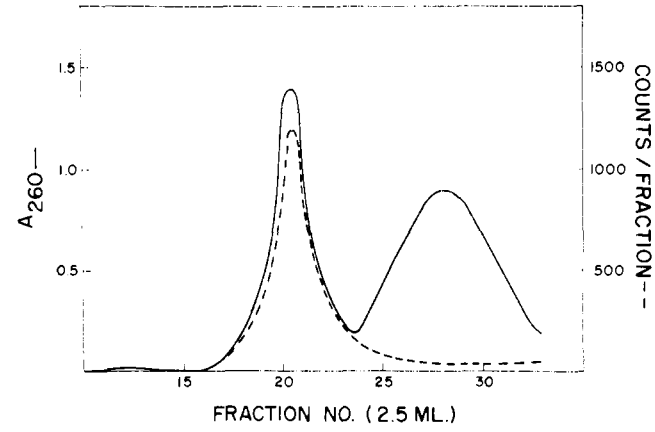

FIGURE 2: Elution profile of sonicated DNA treated arginine-rich nucleohistone chromatographed on Sepharose 4B. The column (1.5 cm × 90 cm) was equilibrated with 0.005 M Tris (pH 8.0) and was monitored by measuring absorbance at 260 nm and by counts per minute of protein incorporated ^{14}C.

brought to the desired Ca^{2+} concentration by way of a four-step gradient from 0.5 mM Ca^{2+} to 2.5 mM Ca^{2+} in the presence of 0.005 M Tris (pH 8.0).

Preparation of ^{14}C-Labeled Chromatin and Arginine-Rich Nucleohistone. ^{14}C-labeled chromatin and arginine-rich nucleohistone were prepared for protein redistribution studies. Purified solubilized chromatin was incubated with ^{14}C-labeled iodoacetamide under conditions (0.005 M Tris (pH 8.5)) which result in the labeling of protein amino groups in chromatin (Crestfield *et al.*, 1963). In Figure 1 a representative elution profile for ^{14}C-labeled chromatin treated with tRNA and chromatographed on a Sepharose 4B column equilibrated with 0.005 M Tris (pH 8.0) is presented. The chromatographic separation was monitored by measuring absorbance at 260 nm and by counts per minute of protein incorporated ^{14}C. The ^{14}C-labeled arginine-rich nucleohistone resulting from this chromatographic separation was incubated with an equal weight of exogenous sonicated DNA for 24 hr in 0.001 M EDTA–0.005 M Tris (pH 8.0) and rechromatographed on Sepharose 4B. An elution profile for sonicated DNA-treated arginine rich nucleohistone as monitored by absorbance at 260 nm and by counts per minute of protein incorporated ^{14}C is presented in Figure 2. Radioactive isotope counting was performed on a Packard 3375 liquid scintillation counter.

Polyacrylamide Gel Electrophoresis. Histones were extracted from the tRNA–histone fraction, the depleted chromatin fraction, and from native chromatin by treatment with 0.25 N HCl. After removal of precipitated nucleotide and nucleoprotein material from these HCl extraction mixtures by centrifugation the supernatants were dialyzed against distilled water and lyophilized to dryness. The histone residues were then dissolved at a concentration of 1 mg/ml in 0.9 M acetic acid–10 M urea; 50-μg samples of extracted histones were separated on 10 cm, 2.5 M urea–polyacrylamide gels by the method of Panyim and Chalkley (1969). Densitometry of Amido Black stained gels using the gel scanning mode of the Unicam Model 1800 spectrophotometer afforded a record of the histone separations. Densitometer tracings of polyacrylamide gel separations of histones extracted from chromatin, chromatin after incubation with tRNA, and the tRNA after incubation with chromatin are shown in Figure 3.

Circular Dichroism Spectroscopy: Circular dichroism spectra were obtained using a Durrum-Jasco J-20 circular dichroism spectrometer calibrated frequently against *d-10*-camphorsulfonic acid (Eastman-Kodak). Measurements were performed at 25° in a N_2 atmosphere using an automatic slit program and an optical path length of 10 mm. The absorbance of chromatin or depleted chromatin samples was 0.7 at 260 nm. Precise concentration of DNA in chromatin and depleted chromatin samples was determined by measuring the absorbance of the sample at 260 nm in 0.1% sodium dodecyl sulfate (Fasman *et al.*, 1971) using an extinction coefficient of 6600 M^{-1} cm^{-1} per nucleotide residue.

Results

An Investigation of Possible Protein Redistribution in Arginine-Rich Nucleohistone. The conditions employed to isolate arginine-rich nucleohistone have been shown not to allow protein redistribution along the DNA during histone extraction (Ilyin *et al.*, 1971; Varshavsky *et al.*, 1972). In order to substantiate these observations ^{14}C-labeled arginine rich nucleohistone prepared as described above was incubated, under the same buffer conditions used to extract the lysine and moderately lysine rich histone fractions, with an equal concentration of exogenous sonicated DNA. If redistribution of remaining histones III and IV and non-histone protein to free DNA regions occurs during the formation or isolation of arginine-rich nucleohistone, then such

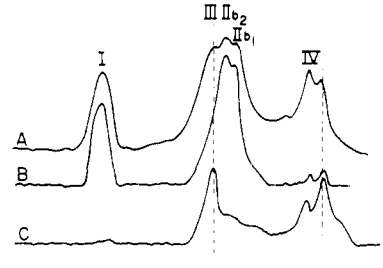

FIGURE 3: Densitometer scans of polyacrylamide gels stained with Amido Black and scanned at 610 nm on a UNICAM Model 1800 spectrophotometer fitted with a gel scanner. Whole histone extracted from calf thymus chromatin (A), histone fractions removed from chromatin by tRNA (B), and histone fractions present in depleted chromatin (C).

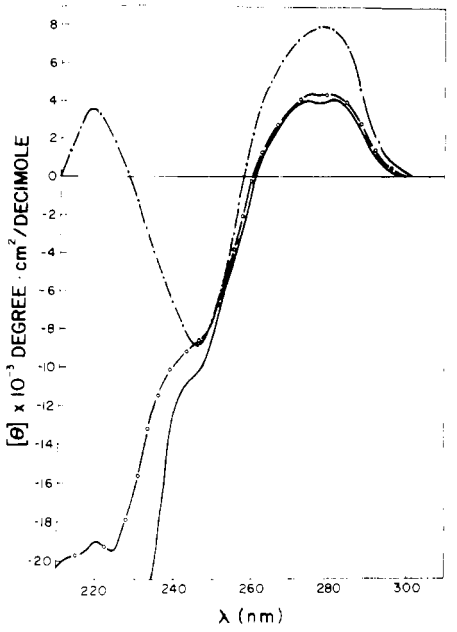

FIGURE 4: Circular dichroism spectra of DNA (●), chromatin (—), and chromatin depleted of all histone fractions except III and IV (○) in 0.005 M Tris (pH 8.0).

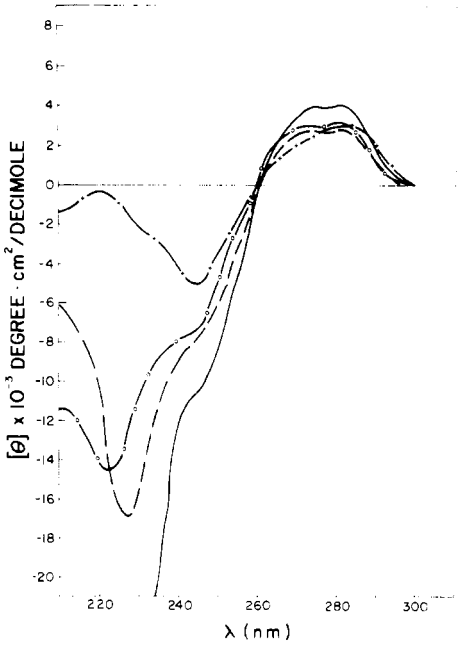

FIGURE 5: Circular dichroism spectra of chromatin in 0.005 M Tris (pH 8.0) (—), chromatin in 0.0025 M CaCl–0.005 M Tris (pH 8.0) (- - -) chromatin depleted of all histone fractions except III and IV, in 0.0025 M $CaCl_2$–0.005 M Tris (pH 8.0) (○), and nucleohistone IV complex prepared by gradient dialysis, in the presence of 0.0025 M $CaCl_2$ (x).

redistribution should reoccur when arginine-rich nucleohistone is treated with exogenous DNA under identical conditions of ionic strength. The elution profile of ^{14}C-labeled arginine-rich nucleohistone incubated with exogenous sonicated DNA for 24 hr in 0.001 M EDTA–0.005 M Tris (pH 8.0) and separated on Sepharose 4B is presented in Figure 2. The complete lack of radioactively labeled protein in the free DNA portion of the elution profile (Figure 2) supports the conclusion that no protein redistribution occurs in arginine-rich nucleohistone.

Circular Dichroism Spectra of DNA, Chromatin, and Arginine-Rich Nucleohistone in 0.005 M Tris (pH 8.0). The circular dichroism spectra of purified chromatin and chromatin after selective histone extraction with tRNA, shown to contain only histones III and IV (Figure 3) and henceforth referred to as arginine-rich nucleohistone, measured in 0.005 M Tris buffer (pH 8.0) are presented with the spectrum of purified DNA in Figure 4. The circular dichroism (CD) spectra of DNA is characterized by a positive maximum at 275 nm ($[\theta]_{275}$ 8.00×10^3), a crossover point at 257 nm, and a negative minimum ellipticity at 246 nm ($[\theta]_{246}$ -8.90×10^3). At higher energy there is a crossover point at 227 nm and a small positive ellipticity maxima at 219 nm ($[\theta]_{219}$ 3.60×10^3). The CD spectrum of chromatin exhibits distinct differences from that of DNA in the entire range of the spectrum. The low-energy portion of the spectrum of chromatin (250–300 nm) is characterized only by contributions from the DNA component of chromatin, whereas the high-energy portion (200–250 nm) is a composite of contributions from chromatin DNA and protein transitions (Wagner *et al.*, 1974). The CD spectrum of chromatin (Figure 4) is red shifted with respect to the DNA CD spectrum in the low-energy region of the spectrum having maximum at 282 nm ($[\theta]_{282}$ 4.00×10^3) and a crossover point at about 260 nm. More significant is the marked decrease in the ellipticity of the positive CD band of chromatin ($[\theta]_{282}$ 4.00×10^3) relative to that of DNA ($[\theta]_{275}$ 8.00×10^3). The CD spectrum of arginine-rich nucleohistone (Figure 3) in the 250–300-nm region of the spectrum is substantially the same as that of native chromatin, with a positive maximum at 282 nm ($[\theta]_{282}$ 4.3×10^3) and a crossover point at 259.5 nm. The high-energy regions of the spectrum of native chromatin and arginine-rich nucleohistone are quite different. This spectral difference is manifested by a decreased negative ellipticity in the negative arginine-rich nucleohistone shoulder at 245 nm ($[\theta]_{245}$ -8.5×10^3) and in the negative arginine-rich nucleohistone band at 222 nm ($[\theta]_{222}$ -19.5×10^3) as compared to native chromatin ($[\theta]_{245}$ -10.5×10^3, $[\theta]_{222}$ -32.5×10^3) (Wagner and Spelsberg, 1971). Since arginine-rich nucleohistone is depleted in the lysine-rich and moderately lysine-rich histones as well as in some non-histone proteins (Ilyin *et al.*, 1971) decreased ellipticity in the protein region of the CD spectrum relative to chromatin is not unexpected.

Circular Dichroism Spectra of Chromatin, Arginine-Rich Nucleohistone, and Reconstituted Nucleohistone IV in the Presence of Calcium Ion. The nucleus of eukaryote cells contains Ca^{2+} at a concentration of approximately 2.5 mM (Mirsky and Osawa, 1961). In order to better simulate the environment of chromosomal material within the cell nucleus CD studies of chromatin and arginine-rich nucleohistone were carried out in the presence of 2.5 mM Ca^{2+}. The CD spectra of chromatin and arginine rich nucleohistone in 2.5 mM Ca^{2+} are presented with the spectrum of reconstituted nucleohistone IV (Wagner and Vandegrift, 1972) and compared to native chromatin in the absence of Ca^{2+} in Figure 5. As has been observed elsewhere (Johnson *et al.*, 1972) the presence of 2.5 mM Ca^{2+} alters the chromatin CD spectrum in the low-energy DNA region causing a decrease in the positive 282-nm band ($[\theta]_{282}$ 2.9×10^3) but does not affect the crossover point significantly. In the high-energy region of the chromatin spectrum a large decrease in negative ellipticity is observed in the presence of 2.5 mM Ca^{2+} ($[\theta]_{245}$ -8×10^3, $[\theta]_{222}$ -17×10^3) suggesting a marked change in chromosomal protein conformation

and/or chromosomal protein–DNA interactions induced by the presence of Ca^{2+}. The CD spectrum of arginine-rich nucleohistone in the presence of 2.5 mM Ca^{2+} (Figure 4) is nearly identical with the spectrum of native chromatin in 2.5 mM Ca^{2+}, in the low-energy region of the spectrum and remarkably similar to the native chromatin spectrum in the high-energy region considering the different protein compositions of these preparations. The more native nuclear milieu provided by the presence of Ca^{2+} increases the similarity of the CD spectra of native chromatin and arginine-rich nucleohistone (Figures 4 and 5). Also presented in Figure 5 is the CD spectrum of a reconstituted histone IV–DNA complex reannealed in a $CaCl_2$ gradient terminated at 5 mM Ca^{2+} and previously published (Wagner and Vandegrift, 1972). This nucleohistone IV complex in 2.5 mM Ca^{2+} is remarkably similar to arginine-rich nucleohistone and native chromatin, in 2.5 mM Ca^{2+}, in the low-energy region of its CD spectrum ($[\theta]_{282}$ 3.0 × 10^3, crossover at 259.5 nm).

Discussion

Chromatin depleted of all histone fractions except the arginine-rich histones III and IV may be prepared by exchanging all other histone species to exogenous tRNA (Figures 1 and 2) (Ilyin *et al.*, 1971). The resulting arginine-rich nucleohistone is formed from native chromatin without any significant redistribution of histones III and IV between different regions of the DNA (Ilyin *et al.*, 1971; Varshavsky *et al.*, 1972) (see Figure 2). Furthermore, histones III and IV are found clustered in arginine-rich nucleohistone with stretches of DNA free of histone separating them (Varshavsky and Georgiev, 1972). The circular dichroism spectrum of arginine-rich nucleohistone in 2.5 mM Ca^{2+} is essentially identical with that of native chromatin in the same solvent (Figure 5). This observation demonstrates that the arginine-rich histones by themselves are capable of maintaining the geometry of the DNA component of native chromatin which gives rise to the unique CD spectrum of chromatin. The remarkable similarity among the low energy (300–250 nm) CD spectra of arginine-rich nucleohistone, native chromatin and a reconstituted histone IV–DNA complex formed in a Ca^{2+} gradient (Wagner and Vandegrift, 1972) all in the presence of 2.5 mM Ca^{2+} (Figure 4) suggests that histone IV alone may be capable of generating and maintaining the unique DNA geometry found in chromatin.

Studies in other laboratories, using X-ray diffraction techniques, have demonstrated that the DNA component of chromatin has a geometry described by a superhelical tertiary structure (100 Å diameter and 120 Å pitch) imposed upon a B-form secondary structure (Pardon *et al.*, 1967; Pardon and Wilkins, 1972). It has been further demonstrated that histone IV alone is capable of maintaining the superhelical structure of DNA in chromatin (Skidmore *et al.*, 1973). In addition to requiring histone IV for supercoiling, Garrett (1971) has demonstrated a requirement for a Ca^{2+} or Mg^{2+} concentration of greater than 1 mM for maintenance of the DNA superhelical structure in chromatin. The marked similarities between our circular dichroism studies and these X-ray diffraction studies suggest a common origin in the molecular structure of the DNA component of chromatin. Indeed, it is suggested that the unique DNA CD spectrum observed for the DNA component of native chromatin may be arising from B-form superhelical DNA.

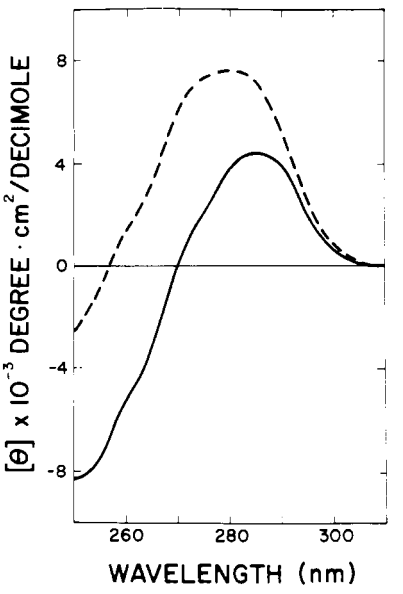

FIGURE 6: Calculated circular dichroism spectra of superhelical and nonsuperhelical B-form DNA. (—) Superhelical B-form DNA; (- - -) nonsuperhelical B-form DNA.

Although the interaction of circular polarized light with dissymmetric molecular systems contains detailed information regarding the molecular geometry of these systems, to date, molecular structure for molecules as complicated as DNA has not been determined from CD data. Circular dichroism studies on covalently closed circular DNA with superhelical densities of approximately −0.03 per 10 base pairs (the negative sign indicates a left superhelix) suggest that the effect of DNA supercoiling on the circular dichroism spectrum of DNA is minimal (Campbell and Lockhead, 1971; Maestre and Wang, 1971). Recent advances in circular dichroism theory (Tinoco, 1968; Johnson and Tinoco, 1969) applied to DNA geometry (Moore and Wagner, 1973, 1974) now make more feasible structural interpretations from DNA CD spectra. Along these lines, we have constructed a computer-simulated model of superhelical DNA of random base sequence. A superhelix or coiled coil may be considered as consisting of a minor helix (the B-form double helix) the axis of which is bent into a major helix (the superhelix) (Crick, 1953). Accordingly, our superhelical model was generated by transforming the coordinates (Johnson and Tinoco, 1969) of B-form double-helical DNA to those of a right superhelix with 120 Å pitch and 100 Å diameter (Pardon *et al.*, 1967; Pardon and Wilkins, 1972). This superhelical DNA differs radically from that of the covalently closed circular DNA studied previously. In addition to being a right superhelix the superhelical density for the proposed DNA superhelix of 0.1 per 10 base pairs is 3 to 4 times more tightly supercoiled than the DNA studied experimentally. The CD spectra calculated for this superhelical DNA is presented in Figure 6 compared to calculated linear B-form DNA. Although the calculated DNA spectrum does not exactly match experimental DNA CD curves, the changes in the calculated spectrum due to supercoiling (Figure 6) closely resemble the differences observed between DNA and the DNA component of native chromatin. The spectrum calculated for the proposed model is, of necessity, representative of a 100% regular supercoiled structure. Thus, the observed circular dichroism spectrum would differ from that calculated depending upon the total

amount of supercoiling present as well as variations in the amount of supercoiling present in different regions of chromatin. Nonetheless, these calculations support the hypothesis that the red-shifted and diminished 282-nm chromatin DNA CD band is the result of a superhelical tertiary structure for DNA in chromatin, a structure postulated by X-ray diffraction studies (Pardon *et al.*, 1962; Pardon and Wilkins, 1972).

We conclude by suggesting that the unique CD spectrum of the DNA component of native chromatin is the result of supercoiling of chromatin DNA by the arginine-rich histones, probably specifically histone IV, aided by the presence of Ca^{2+}.

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