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The Role of Histones in the Conformation of DNA in Chromatin as Studied by Circular Dichroism†

Rex P. Hjelm, Jr.,‡ and Ru Chih C. Huang*

ABSTRACT: This paper is an investigation of the role of histone in the conformation of DNA in chromatin. Quantitative acrylamide gel electrophoresis of proteins from chromatins isolated from various tissues and dissociated with solutions of sodium chloride–5 mM phosphate of ionic strengths 0.6, 1.0, 1.2, and 3.0 indicates that the response of each chromatin to dissociation is different as to the amount and type of histone removed at each of the sodium chloride concentrations, the exception to this being the unique removal of histone I from all the chromatins with 0.6 ionic strength solutions. Correlation of these observations with the circular dichroism (CD) above 260 nm of each of the depleted nucleoproteins shows that no change occurs in the CD of the chromatins with removal of the lysine-rich histone I; furthermore, the changes which occur with removal of the other (slightly lysine- and arginine-rich) histones are found to be approximately linear with the amount, not the type, of total histone in the depleted nucleoprotein sample. This suggests that the secondary structure of chromatin DNA is determined equally and independently by each of the slightly lysine-rich and arginine-rich histone fractions. This point is supported by an inverse, linear correlation observed between the magnitude of the CD above 260 nm of depleted nucleoproteins and the amount of slightly lysine- and arginine-rich histones bound to a DNA as estimated by analysis of thermal denaturation data. In contrast, the data show that the nonhistone proteins do not effect the CD of DNA in chromatin although they do bind the DNA.

Isolated chromosomes of higher plants and animals—usually studied in the form isolated from interphase nuclei, chromatin—are found to consist of DNA, protein, and some RNA. The major constituent of the protein component is the histone proteins, a set of five small, basic proteins which are characterized by their high content of lysine and arginine, and are ultimately bound to the DNA (Stellwagen and Cole, 1969; Georgiev, 1969; Hearst and Botchan, 1970; DeLange and Smith, 1971; Elgin *et al.,* 1971; Huang and Hjelm, 1974). The interactions of histones and their relationship to the structure of the chromosome have been the focus of many types of physical studies on the chromosome.

The integration of DNA into chromatin involves a change in the secondary structure of the DNA, and it is usually assumed that this is a direct result of the constraints placed on the DNA topology by the folding or coiling of the DNA duplex into a chromatin fibril 100–250 Å in diameter (Ris and Kubai, 1970; DuPraw, 1970). One powerful method of probing the secondary structure of chromosomal DNA is circular dichroism (CD). The region of the chromatin spectrum above 250 nm reflects largely the conformation of the DNA component, though we have shown that RNA and nonhistone protein chromophores can contribute a small amount to the CD in this region (Hjelm and Huang, 1974).

It appears well established that the histone proteins are the major determinants of the structure of DNA in chromatin. This has been demonstrated, in part, by removing the histones either partially or completely with solutions of different concentrations of NaCl (Ohlenbusch *et al.,* 1967). As the histones are removed the properties of the complexed DNA become more and more like those of DNA free in solution. Since somewhat selective removal of the different histone fractions occurs at various NaCl concentrations, workers have attempted to determine by CD which histone, if any, has the greatest role in determining the secondary structure of DNA in chromatin (Henson and Walker, 1970b; Simpson and Sober, 1970; Wagner and Spelsberg, 1971). Similar studies have been carried out on the hydrodynamic (Henson and Walker, 1970a; Ohba, 1966) and dye binding (Kleiman and Huang, 1971; Angerer and Moudrianakis, 1972) properties of chromatin. The results of these and the CD studies have been interpreted as being due to the association of different histone fractions with distinct physical characteristics of chromatin. The CD studies show that the removal of the lysine-rich histone I does not affect

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‡ Present address: Biophysics Laboratories, Department of Physics, Portsmouth Polytechnic, Gun House, Hampshire Terrace, Portsmouth P01 2QG, England.

the ellipticity of the chromatin, but, as the other histones are removed at higher salt concentrations, the ellipticity is seen to increase, until, with the removal of all the histones, the ellipticity of the chromosomal DNA is essentially that of free DNA. There is disagreement as to which histone, if any, is most responsible for the observed secondary structure of DNA in chromatin. For example, the results of Henson and Walker (1970b) led them to conclude that histone fractions III (argine-rich), IIb1, and IIb2 (slightly lysine-rich) are responsible. Simpson and Sober (1970), on the other hand, have concluded that only the slightly lysine-rich histones, IIb1 and IIb2, are the major determinants of chromosomal DNA structure. In addition, Wagner and Spelsberg (1971) indicate that histone IV-DNA interactions may be the most important of the structure of the DNA.

What is the source of these divergent views? Bartley and Chalkley (1972) have pointed out that in calf thymus, the system used by Henson and Walker (1970b), degradation of histone may occur during NaCl dissociation. Furthermore, when centrifugation is used to separate the partially dissociated nucleoproteins from the dissociated components, as done by Wagner and Spelsberg (1971), some of the removed histones may sediment with the nucleoprotein due to the aggregation of histones in high salt. Bartley and Chalkley (1972) also point out that although the order of removal of histone with increasing NaCl concentration is such that the lysine-rich histones are removed at lowest salt, followed by the slightly lysine-rich histones at higher concentrations, followed finally by the dissociation of the arginine-rich histones at the highest salt concentrations, the quantitative removals overlap. Thus, the removal of one set of histones may be generally accompanied by the removal of substantial amounts of other types. Previous work had failed to detect this by quantitative gel electrophoresis. Bartley and Chalkley (1972) have also shown that the amount of a given type of histone removed at any salt concentration is dependent on the method of dissociation. Added to this is the possibility that the quantity of each histone removed may be different for chromatins isolated from different sources. Also, nonhistones are removed concomitantly with the histone proteins (Kleiman and Huang, 1971), and these too may contribute to the conformation properties of the chromosomal DNA. Thus, without careful consideration of each of these factors, the interpretation of the results for the CD of chromatin will be handicapped.

Many of the questions and discrepancies of the earlier work may be resolved by control of histone degradation and by analysis of the histone content of each sample. The use of different sources of tissue for chromatin—each being dissociated in the same manner—may also help clear up the reasons for the above differences. In these experiments we dissociate chromatins from five tissues: calf thymus, pig cerebellum, chick embryo brain, chicken erythrocyte, and the mucine solid tumor, myeloma K41. We observe that the results for each chromatin are indeed different, but that all the observed differences in the CD are the result of variation in the extractability of histone fractions in various tissues. The data indicate that a linear correlation exists between the observed ellipticity and the amount of histone present in the sample. We agree with the results of the other authors that the removal of the lysine-rich histone causes litle change in the CD of chromatin DNA, and that this may be due to the different role of the lysine-rich histone in chromatin structure.

Experimental Section

Materials

Calf thymus, pig cerebellum, chicken blood, chick embryo brain, and myeloma K41 were obtained as described elsewhere (Hjelm and Huang, 1974).

Methods

Isolation of Nuclei and Chromatin. Nuclei were isolated by the methods described elsewhere (Hjelm and Huang, 1974). Chromatin was isolated by the method of Huang and Huang (1969). Sodium sulfite (0.05 M, pH 8.0) was added to the solutions containing 0.075 M NaCl and 0.015 M EDTA (pH 8.0) used for the isolation of calf thymus chromatin to prevent degradation of histones (Bartley and Chalkley, 1970).

Dissociation of Chromatin. Chromatin was dissociated in solutions of ionic strengths 0.6, 1.0, 1.2, and 3.0. The solutions contained 5 mM phosphate (pH 6.8) and NaCl to the desired ionic strength. Dissociation was carried out by overnight dialysis of sheared chromatin (Waring Blendor, 80 V [Variac], 1 min) against large volumes of dissociation solution. Sodium sulfite (0.05 M, pH 6.8) was added to the solutions used for dissociation of calf thymus chromatin to prevent degradation (Kleiman and Huang, 1972). The NaCl concentration was reduced accordingly. After dissociation the samples were cleared by centrifugation at 10,000 rpm for 15 min in a Sorvall SS-34 rotor. The sample was applied to a Bio-Gel A-50m column (Bio-Rad, Richmond, Calif.) equilibrated in a solution identical with that used to effect the dissociation. The column was monitored at 230 and 260 nm. Where sulfite was present the protein elution was monitored by a trichloroacetic acid turbidity technique (Bonner *et al.*, 1968a). Dissociated samples are referred to as depleted nucleoproteins. Alternatively, the nomenclature nucleoprotein for the depleted nucleoprotein or extract for the dissociated fraction, respectively, is used, followed by the ionic strength of the solution used for dissociation. Thus, nucleoprotein 1.0 is 1.0 M salt-dissociated chromatin. All procedures were carried out at 4°.

Quantitative Determination of Chromatin Components. Protein content of the chromatin and depleted nucleoprotein samples was determined by the method of Lowry *et al.* (1951). DNA determinations by the diphenylamine method of Burton (1956) were carried out on the hot 5% perchloric acid (70°, 10 min) soluble fraction of chromatin and depleted nucleoproteins.

Qualitative Analysis of Proteins. Proteins were removed from chromatin and depleted nucleoprotein by 5 M urea–2 M NaCl as described elsewhere (Hjelm and Huang, 1974). Dialyzed and lyophilyzed samples were dissolved in 0.9 M acetic acid and were electrophoresed on 9-cm, 15% polyacrylamide gels (after Panyim and Chalkley, 1969). The gels were stained with Buffalo Blue-Black for 4 hr and destained by diffusion into 7% acetic acid. The amount of stain in each histone band was determined by scanning the gel with a Joyce Comparitor. The trace for each band was cut out and weighed. The amount of dye in each band was normalized with the amount of stain in the band corresponding to histone IV.

Measurement of Circular Dichroism. Chromatin and depleted nucleoprotein were exhaustively dialyzed against 0.7 mM phosphate (pH 6.8). Concentrations were adjusted to 1.7 optical density (OD) units at 260 nm. Unless otherwise

TABLE I: Protein to DNA Mass Ratio of Chromatins Dissociated by Solutions of Various Ionic Strengths.[a]

Source	Sample					
	CHR[b]	NPLS[c]	NP[d] 0.6	NP 1.0	NP 1.2	NP 3.0
Chick embryo brain	3.3 ± 0.3	2.1 ± 0.3	1.8 ± 0.3	1.4 ± 0.2	1.0 ± 0.2	0.3 ± 0.1
	1.00	0.66 ± 0.07	0.56 ± 0.08	0.42 ± 0.04	0.29 ± 0.05	0.10 ± 0.02
	(17)	(6)	(13)	(6)	(4)	(5)
Pig cerebellum	3.1 ± 0.3	2.6 ± 0.2	2.1 ± 0.2	1.8 ± 0.2	1.4 ± 0.2	0.8 ± 0.1
	1.00	0.86 ± 0.04	0.72 ± 0.08	0.61 ± 0.10	0.41 ± 0.03	0.23 ± 0.02
	(17)	(12)	(6)	(5)	(4)	(2)
Myeloma	2.3 ± 0.4	1.9	1.5 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	0.2
	1.00	0.79	0.73 ± 0.07	0.45 ± 0.06	0.28 ± 0.04	0.09
	(9)	(1)	(3)	(4)	(5)	(2)
Calf thymus	1.9 ± 0.3	1.9	1.4 ± 0.2	0.93 ± 0.14	0.85 ± 0.08	0.03 ± 0.1
	1.00	0.86	0.72 ± 0.08	0.49 ± 0.10	0.44 ± 0.05	0.13 ± 0.04
	(12)	(1)	(5)	(8)	(7)	(4)
Chicken erythrocyte	2.2 ± 0.1		1.7 ± 0.1	1.2 ± 0.3	0.8 ± 0.1	0.2 ± 0.1
	1.00		0.75 ± 0.06	0.54 ± 0.12	0.34 ± 0.02	0.06 ± 0.02
	(7)		(5)	(5)	(2)	(2)

[a] The protein to DNA mass ratio (milligram/milligram) for each sample is given in the first line under each sample and source. The amount of protein in each sample relative to the protein content of the respective chromatin is shown in the second line. The number of determinations is shown in parentheses. [b] Chromatin. [c] Nucleoprotein low salt. [d] Nucleoprotein, followed by ionic strength.

stated, the spectra were measured at 4°. Spectra were recorded on a Cary 60 recording spectropolarimeter fitted with a 6003 CD attachment. Cells were 10 mm in pathlength.

Thermal Denaturation. Samples exhaustively dialyzed against 2.4×10^{-4} M EDTA were adjusted to 1.2 OD_{260} units and melted at 0.4° for 1 min.

Calculations. CD data were reduced as described elsewhere (Hjelm and Huang, 1974). Data are reported plus or minus standard deviation. Thermal denaturation data are reported as per cent hyperchromicity at 260 nm. Points are taken at temperature increments of 0.5°. Derivative curves of the melt profiles are calculated by a sliding-point least-squares procedure outlined by Ansevin and Brown (1971). In our calculations, nine points of the melting curve are fit to a second-order polynomial and the instantaneous derivative calculated for the central point.

Results

Dissociation of Chromatins with Solutions of High Ionic Strength. We have demonstrated that exposure of chromatin to NaCl and 5 mM phosphate solutions of ionic strength as high as 0.35 removes only nonhistone proteins and RNA (Hjelm and Huang, 1974). At higher concentrations, the histone proteins are seen to be removed (Ohlenbusch *et al.*, 1967; Bartley and Chalkley, 1972). The amount and type of histone removed are dependent upon the NaCl concentration of the dissociating solution, and the dissociation at any given concentration is dependent upon the chromatin treated. For our systems this is demonstrated in Tables I and II and Figure 1. In all cases the lysine-rich histone I is removed completely by 0.6 M NaCl, as are the histones unique to pig cerebellum and chicken erythrocyte, I^0 and V, respectively (Figures 1, parts I and III). In chicken erythrocyte chromatin a small amount of the slightly lysine-rich histone IIb1 is also removed at this ionic strength (Figure 1, part III).

TABLE II: Fraction of Histone Remaining after NaCl Extraction of Chromatins.[a]

Source	Histone Fraction	NP 0.6[c]	NP 1.0	NP 1.2
Chick embryo brain	I	0.0	0.0	0.0
	III	1.00	0.75	0.62
	IIb2	1.00	0.28	0.0
	IIb1	1.00	0.26	0.0
	IV	1.00	1.00	1.00
	Total[b]	1.00	0.49	0.30
Pig cerebellum	I	0.0	0.0	0.0
	III	1.00	1.00	0.95
	IIb2	1.00	0.34	0.0
	IIb1	1.00	0.25	0.0
	IV	1.00	1.00	1.00
	Total[b]	1.00	0.59	0.39
Calf thymus	I	0.0	0.0	0.0
	III	1.00	0.53	0.60
	IIb2	1.00	0.10	0.11
	IIb1	1.00	0.17	0.38
	IV	1.00	1.00	1.00
	Total[b]	1.00	0.35	0.41
Chicken erythrocyte	I	0.0		
	III	1.00		
	IIb2	1.00		
	IIb1	0.73		
	IV	0.73		
	Total[b]	0.80		

[a] Determined by quantitative gel electrophoresis as indicated in the Experimental Section. [b] Fraction of total histones II, III, and IV remaining on chromatin extracted with the different salt solutions. [c] Nucleoprotein, followed by ionic strength.

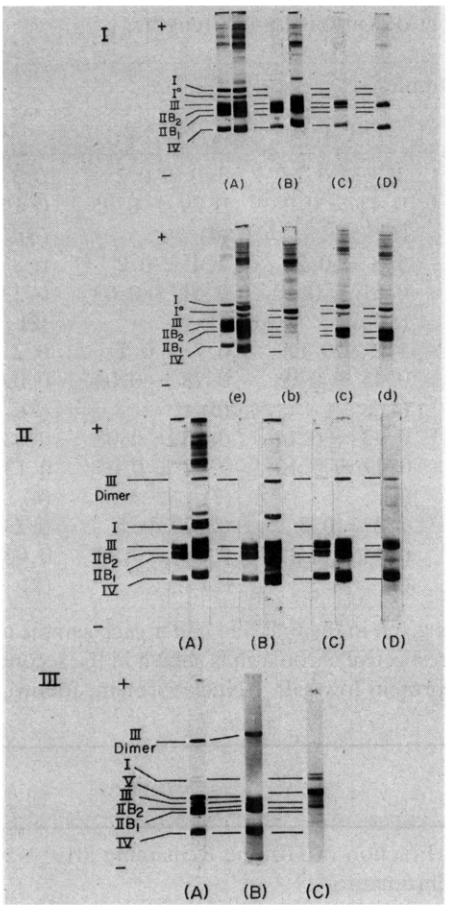

FIGURE 1: Urea–polyacrylamide gels of protein of different loading from chromatin and depleted nucleoproteins. (I) Pig cerebellum: (A) chromatin; (B) nucleoprotein 0.6; (C) nucleoprotein 1.0; (D) nucleoprotein 1.2; (a) extract 3.0; (b) extract 0.6; (c) extract 1.0; (d) extract 1.2. (II) Chick embryo brain: (A) chromatin; (B) nucleoprotein 0.6; (C) nucleoprotein 1.0; (D) nucleoprotein 1.2. (III) Chick erythrocyte: (A) chromatin; (B) nucleoprotein 0.6; (C) extract 0.6.

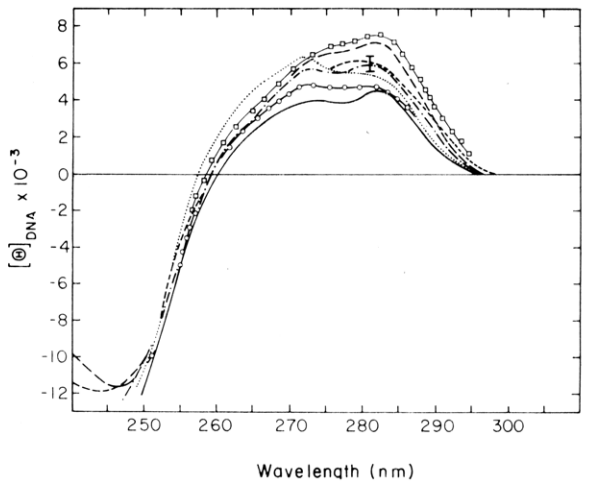

FIGURE 2: Circular dichroism of chick embryo brain chromatin and depleted nucleoproteins: (···) chromatin; (○) nucleoprotein low salt; (—) nucleoprotein 0.6; (- · -) nucleoprotein 1.0; (- - -) nucleoprotein 1.2; (– – –) nucleoprotein 3.0; (□) DNA. Error bar indicates standard deviation and applies to all spectra shown.

At higher NaCl concentrations the slightly lysine-rich histones (Table II) are seen to be removed in all the samples studied. Murray *et al.* (1970) have demonstrated that this is also the case for chicken erythrocyte chromatin. With the exception of calf thymus, removal of these histones is complete at 1.2 M NaCl. Histone III is partially removed with the slightly lysine-rich histone in chick embryo brain and calf thymus (Table II) and larger amounts are seen to be removed in 1.2 ionic strength solution. In no case did we detect the removal of the arginine-rich histone IV (see Figure 1) in any of the solutions at or below ionic strength 1.2. All detectable histones are seen to be removed by 3.0 M NaCl. A substantial amount of protein is seen to be present after the removal of the histone proteins (Table I). These are termed the residual nonhistone proteins.

NaCl extraction of calf thymus chromatin has been observed to be associated with two serious problems, histone degradation and aggregation (Bartley and Chalkley, 1972). Histone aggregation is not a problem in our case, since gel filtration, rather than centrifugation, is used to effect the separation of the dissociated components from the depleted nucleohistone. In calf thymus, degradation is effectively inhibited by the addition of 0.05 M sodium sulfite–bisulfite

TABLE III: Ellipticities at 283 nm of Nucleoproteins.

Source	CHR[a]	NPLS[b]	NP 0.6	NP 1.0	NP 1.2	NP 3.0
Chick embryo brain	5100 ±480 (18)	4410 ±490 (10)	4050 ±640 (14)	5580 ±760 (6)	6150 ±380 (4)	7020 ±490 (6)
Pig cerebellum	4670 ±680 (17)	4270 ±480 (16)	3970 ±340 (5)	4690 ±630 (6)	6090 ±980 (7)	7700 (3)
Myeloma	4620 ±660 (9)	4455 (1)	3810 ±430 (4)	5650 ±280 (4)	7070 ±790 (5)	7580 ±540 (4)
Calf thymus	4650 ±610 (7)	4275 (1)	4290 ±440 (6)	6350 ±670 (7)	6150 ±300 (7)	7580 ±610 (5)
Chicken erythrocyte	5310 ±570 (6)		3620 ±550 (5)	5610 ±280 (4)	5210 ±190 (2)	7600 ±750 (3)

[a] See footnote of Table I for abbreviation. [b] Nucleoprotein 0.2–0.35.

(pH 6.8) to the dissociation medium (Kleiman and Huang, 1972). Histone degradation is not a problem in pig cerebellum nucleoproteins (Figure 1). Even overloaded gels (Figure 1) fail to detect possible degradation products. Some degradation may occur, however, in the dissociated histones (Figure 1), but this is of no significance to the results of the CD studies of the dissociated chromatin. Although no detectable degradation is seen associated with chick embryo brain chromatin (Figure 1, part II), some possible histone degradation products are seen in heavily overloaded gels of the depleted nucleoproteins (Figure 1, part II). This possible degradation is small and probably does not affect our results. Extensive degradation of the dissociated chick histones is observed (not shown).

Physical Properties of Chromatin Dissociated with High Concentrations of NaCl–5 mM Phosphate. Dissociation of chromatin with solutions of NaCl–5 mM phosphate of ionic strengths 0.6–3.0 causes progressive changes in the nucleoprotein CD spectra. Figure 2 illustrates the changes which occur in the CD of chick embryo brain chromatin; these are characteristic of the effect of dissociation on the other chromatins studied (Table III). Included in Figure 2 is the spectrum of chromatin extracted with low salt (nucleoprotein low salt; Hjelm and Huang, 1974). Treatment of chromatin with 0.6 ionic strength dissociation solution results in little change in the spectrum over that produced by removal of a portion of the RNA and nonhistone proteins (Hjelm and Huang, 1974). A small decrease in the ellipticity may occur, but the significance of this is difficult to assess. When higher concentrations of NaCl are used to effect the dissociation the CD increases. Titration of the chromatin with 3.0 M NaCl results in a spectrum that is identical with that of free DNA. These observations are similar to those of other laboratories (Henson and Walker, 1970b; Simpson and Sober, 1970; Wagner and Spelsberg, 1971; Fric and Sponar, 1971). The data for ellipticities at 283 nm for all the samples are tabulated in Table III. It is obvious from these data that, although the different chromatins behave similarly to dissociation by each NaCl concentration, the response for each chromatin is significantly different.

The details of how each system responds to dissociation are summarized in Figure 3. The points are calculated by taking the ratio of ellipticities at 283 nm of samples partially depleted at one concentration of NaCl to those of samples dissociated by a solution of the next lowest ionic strength. Thus, points at 0.6 M are the ratio $[\theta]^{283}_{DNA,NP\,0.6}/[\theta]^{283}_{DNA,CHR}$ or at 1.0 M the ratio $[\theta]^{283}_{DNA,NP\,1.0}/[\theta]^{283}_{DNA,NP\,0.6}$.[1] Comparisons in this manner are made only between samples made from the same chromatin preparation; this reduces some of the variability. In most cases we agree with the results of Henson and Walker (1970b) and Simpson and Sober (1970) that the largest changes occur in the CD of the chromatins when they have been dissociated with 1.0 M NaCl. The obvious exception is pig cerebellum nucleoprotein 1.0 which shows little or no change over that obtained with 0.6 M NaCl; in this sample treatment with 1.2 M salt produces the largest change. It is clear that the chromatins differ in the ellipticity change that occurs between samples which are dissociated at 1.2 M NaCl and samples totally stripped of histones at 3.0 M NaCl.

It is evident that there is no unifying, simple correlation of the observed ellipticities with either total protein content of the sample (Tables I and III) or NaCl concentration

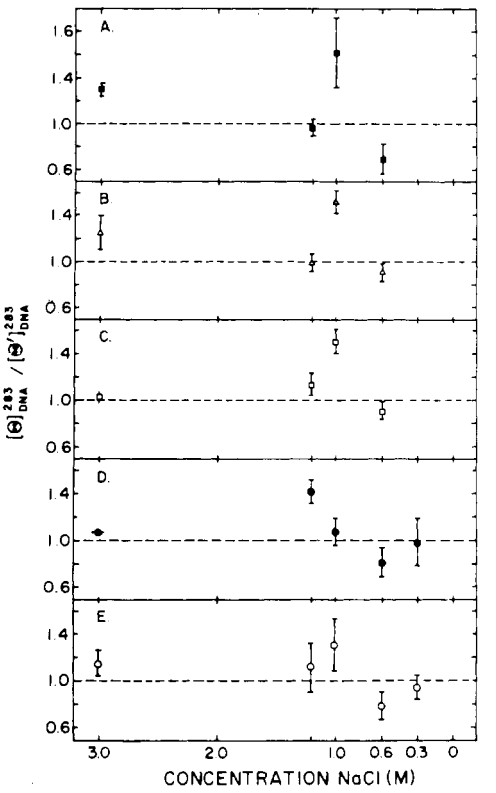

FIGURE 3: Ratio of ellipticities at 283 nm of dissociated chromatins: (A) calf thymus; (B) chicken erythrocyte; (C) myeloma; (D) pig cerebellum; (E) chick embryo brain. Plotted data are taken from samples dissociated with solutions of differing ionic strengths. $[\theta]^{283}_{DNA}/[\theta']^{283}_{DNA}$ ratios are taken of the ellipticity of one sample at 283 nm over the ellipticity at 283 nm of the sample dissociated in a solution of the next lower ionic strength in the same experiment. For example, the points plotted at 0.6 M are the ratios of the ellipticity at 283 nm of nucleoprotein 0.6 over that of chromatin; the point plotted at 1.0 M NaCl is the ratio of the ellipticity at 283 nm of nucleoprotein 1.0 over that of nucleoprotein 0.6; at 1.2 M are the ratios of the ellipticity at 283 nm of nucleoprotein 1.2 over that of nucleoprotein 1.0; and finally at 3.0 M are the ratios of the ellipticity at 283 nm of nucleoprotein 3.0 over that of nucleoprotein 1.2. Error bars show standard deviation.

used to effect the dissociation. The sole exception to this is 0.6 M NaCl dissociation.

We have seen that the amount of total histone and amount of any given type of histone dissociated at any given salt differ among the various chromatins. A plot of ellipticity *vs.* the fraction of slightly lysine-rich (IIb1 and IIb2) plus arginine-rich (III and IV) histones remaining on the partially dissociated nucleohistone (Table II) is shown in Figure 4. The data strongly suggest an inverse and linear correlation between the ellipticity of the depleted nucleoprotein and the fraction of these histones remaining (Figure 4).

Thermal denaturation profiles and derivatives of chick embryo brain chromatin and partially depleted nucleoproteins are shown in Figure 5. Li and Bonner (1971) have resolved the derivatives of the thermal denaturation profiles of calf thymus chromatin and nucleoproteins into several melting bands—each belonging to a melting transition of DNA in a different environment—corresponding to DNA unbound by proteins or bound to histones or nonhistone proteins. Chick embryo brain chromatin and depleted nucleoproteins are seen to have several such melting bands (Figure 5 and Table IV). The two melting bands (bands 1 and 2) at the highest temperatures, T_m at approximately 73 and 83°, have been assigned to the melting of DNA associated with

[1] Abbreviations used are: CHR, chromatin; NP, nucleoprotein.

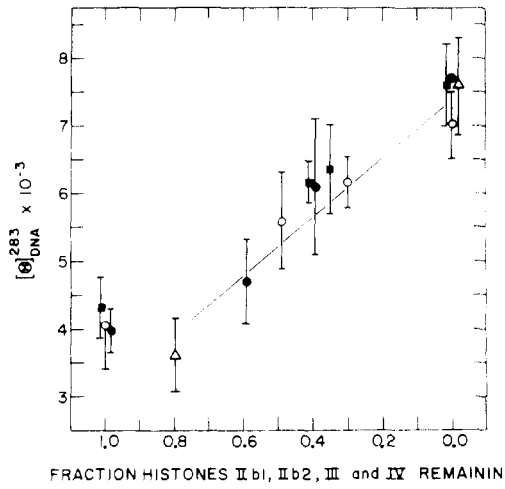

FIGURE 4: A plot of ellipticity *vs.* the fraction of slightly lysine-rich histones (IIb1, IIb2) and arginine-rich histones (III, IV) remaining on the depleted nucleoproteins: (open circles) chick embryo brain; (filled circles) pig cerebellum; (filled squares) calf thymus; (open triangles) chicken erythrocytes.

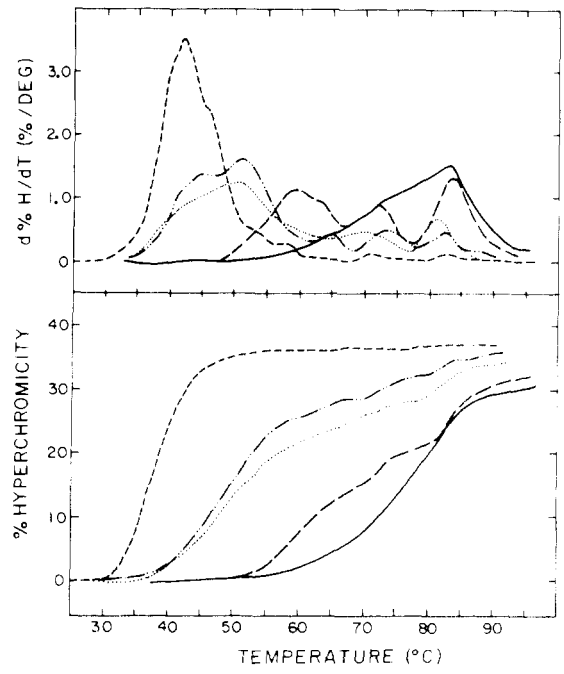

FIGURE 5: Thermal denaturation of chick embryo brain chromatin and depleted nucleoproteins: (—) chromatin; (– – –) nucleoprotein 0.6; (• • •) nucleoprotein 1.0; (• – •) nucleoprotein 1.2; (– – –) nucleoprotein 3.0. Lower graph shows measured hyperchromicities of samples. Upper graph shows calculated derivatives of hyperchromicities.

histones (Li and Bonner, 1971). It has been demonstrated that the area under these two bands (equal to the contribution of the bands to the total hyperchromicity of the sample) is proportional to the amount of histone in the nucleoprotein (Li, 1973; Li *et al.*, 1973). We observe that the contribution of these bands to the total hyperchromicity decreases as histones are removed. Concomitant with this is an increase in the bands assigned to the melting of free DNA (Figure 5 and Table IV; Li and Bonner, 1971). A comparison of the areas under the 73 and 83° melting bands in the nucleoprotein 0.6, nucleoprotein 1.0, nucleoprotein 1.2, and nucleoprotein 3.0 samples (Table IV) with the fraction of slightly lysine-rich and arginine-rich histones remaining in these samples (Table II) indicates a good correspondence between the two, in support of the contention of Li (Li, 1973; Li *et al.*, 1973). A plot of the areas under these bands *vs.* the ellipticity at 283 nm of the chick embryo brain samples gives a linear relationship among the points corresponding to samples nucleoprotein 0.6 through nucleoprotein 3.0 (Figure 6). This further supports the view that the secondary structure of the DNA in chromatin and depleted nucleoproteins is not due to the type of slightly lysine-rich or arginine-rich histone remaining on the sample, but rather it is due to the amount of these histones remaining.

Discussion

The data presented here tie together the observations of earlier work on the effects of dissociation of chromatins with solutions of various concentrations of NaCl and 5 mM phosphate (pH 6.8) (Henson and Walker, 1970b; Simpson and Sober, 1970; Wagner and Spelsberg, 1971). Whereas in previous work the investigators were lead to suggest that specific—but in each case different—histone fractions are responsible for the secondary conformation of DNA in chromatin, as measured by CD, we find that among the slightly lysine-rich and arginine-rich histones each histone molecule has an equal role in the determination of DNA secondary conformation. Entire histone fractions may differ in their total contribution, but only to the extent that they constitute a larger or smaller fraction of the total histone complement of the chromosome. This conclusion is derived from the apparently linear relationship between the total slightly lysine- and arginine-rich histone content of the various dissociation samples and the measured ellipticity (Figure 4). This relationship is supported by the observation that the amount of DNA melting in the hyperchromicity bands assigned to DNA bound to histone (proportional to the amount of histone bound to DNA in the sample; Li, 1973; Li *et al.*, 1973) in chromatin treated with 0.6 or higher molarity NaCl is linearly correlated with the ellipticity at 283 of that sample (Figure 6). The different conclusions of the earlier observations seem to be the result of variation in the efficiency of removal of the slightly lysine-rich and arginine-rich histones from chromatins isolated from different sources. Perhaps the nonhistone proteins are responsible for this variability. Other sources of variance arise from degradation and aggregation of histones (Bartley and Chalkley, 1972).

It is clear from our observations, as it is in the earlier work (Henson and Walker, 1970b; Simpson and Sober, 1970; Wagner and Spelsberg, 1971), that the removal of the fraction of the total histone complement corresponding to the lysine-rich histones causes no significant change in the CD of the positive ellipticity region. This observation is consistent with the results of other physical measurements which show no change with removal of the lysine-rich histones. These include measurements of chromatin sedimentation, intrinsic viscosity, frictional factor (Henson and Walker, 1970a), flow dichroism, rotatory diffusion (Ohba, 1966), actinomycin D binding (Kleiman and Huang, 1971), and X-ray diffraction (Bradbury *et al.*, 1972; Skidmore *et al.*, 1973). It is not possible on the basis of these measurements alone to determine if the immutability of these physical properties to depletion of histone I is due to quantitative or qualitative effect. However, biochemical data for histone I on its structure (Rall and Cole, 1971), heterogeneity (Kinkade and Cole, 1966a,b; Bustin and Cole, 1969; Rall

TABLE IV: Thermal Denaturation of Chick Embryo Brain Chromatin and Depleted Nucleoproteins.

Sample	Band	T_m, °C	% Total Hyperchromicity	A_{1+2}/A_{1+2}^{NPLS} [a]
CHR	1	83	90	106
	2	73		
	3	63	10	
NPLS	1	83	83	100
	2	73		
	3	63	17	
NP 0.6	1	83	60	72
	2	72		
	3	63	10	
	4	59	30	
NP 1.0	1	81	30	36
	2	70		
	3	63	7	
	4	50	63	
	5	43		
NP 1.2	1	83	21	25
	2	73		
	3	60	6	
	4	50	73	
	5	43		
NP 3.0	1			0
	2			
	3			
	4	41	100	
	5			

[a] Area under melting bands 1 + 2 normalized to the area of these bands in low salt extracted (ionic strength 0.2–0.35) chick embryo brain chromatin (Hjelm and Huang, 1974). Numbers are expressed as per cent.

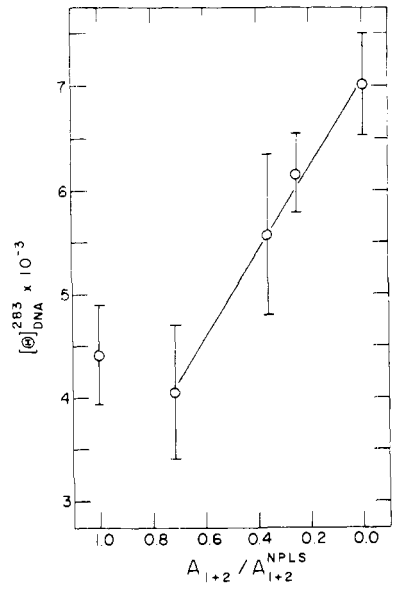

FIGURE 6: Ellipticity of chick embryo brain chromatin at 283 nm *vs.* the fraction total hyperchromicity in melting bands 1 and 2. A_{1+2}/A_{1+2}^{NPLS} is the area under bands 1 + 2 normalized to the area of these bands in nucleoprotein low salt.

and Cole, 1971), species specificity (Panyim *et al.*, 1971; Bustin and Stollar, 1972, 1973), function in the cell cycle (Oliver *et al.*, 1972; Marks *et al.*, 1973; Louie and Dixon, 1973; Bradbury *et al.*, 1973b), and binding to DNA (Kleiman and Huang, 1972; Bartley and Chalkley, 1972) in comparison to analogous data for other histones indicate a unique role for the lysine-rich histone. Evidence that the removal of histone I from chromatin does affect certain characteristics of chromatin such as condensation (Littau *et al.*, 1965; Bradbury *et al.*, 1973a) and ethidium bromide binding (Angerer and Moudrianakis, 1972) indicates that some structural features of chromatin are affected by histone I. Some of these considerations have lead Littau *et al.* (1965), Louie and Dixon (1973), and Bradbury *et al.* (1973a) to suggest that the role of this histone is to cross-link the chromatin fibrils; it is not, according to this suggestion, involved in the folding or coiling of the DNA in the basic chromatin fibril. This is supported by the observation that the removal of the lysine-rich histone results in the apparent release of DNA melting in the histone bands (1 and 2; Figure 5) to those bands assigned to the melting of "free" DNA without affecting the CD (a situation similar to that observed for the low salt-extractable nonhistone proteins; Hjelm and Huang, 1974).

The linear relationship observed between the ellipticity and slightly lysine- and arginine-rich histone to DNA mass ratio indicates that these histones act independently in determining the unique secondary structure of DNA in chromatin. If histone to histone interactions are involved, these data indicate that types of histones which interact are restricted to the slightly lysine-rich histones. This consideration has important implications for the way that histones interact to produce the coiling or folding of DNA into the 100-Å fibril, if it is true that the altered secondary conformation of DNA in chromatin as measured by CD is a reflection of the higher order structure of packed DNA. It should be pointed out that this connection is not necessarily the case, especially once the chromatin is dissociated and possibly denatured by NaCl. For example, association of DNA with polylysine (Shapiro *et al.*, 1969; Carroll, 1972; Chang *et al.*, 1973) or lysine-rich histone (Fasman *et al.*, 1970; Sponar and Fric, 1972; Yu and Li, 1973) at the correct protein to DNA ratio produces an altered CD of DNA much like that observed for chromatin, but which does not produce a structure anything like the fibril of chromatin. Furthermore, salts, such as NaCl of the proper concentration in aqueous solution, can cause the DNA to mimic the CD of chromatin DNA (Studdert *et al.*, 1972; Henson and Walker, 1970b). Here there is no correlation with tertiary or higher order structure. Furthermore, Li and coworkers have found that the CD difference of DNA bound to polylysine (Chang *et al.*, 1973) and protamines (Yu and Li, 1973) from the CD of isolated DNA is linearly proportional to the amount of DNA bound by the polylysine or protamine—as we observe here for histones in partially depleted nucleoproteins. This gives further support to the possibility that the CD of histone-bound DNA may be a reflection of a general property of cation-bound DNA, not to a secondary structure specifically associated with chromatin DNA.

There is evidence, however, that the release of constraints which hold the DNA in the altered secondary structure corresponds to the disruption of the DNA packing in the chromatin fibril. We have previously recounted the characteristics which change as the chromatin is dissociated with increasing concentrations of NaCl. The shapes of the curves plotted for the intrinsic viscosity and frictional factor *vs.*

TABLE V: Nonhistone to DNA Mass Ratios of Nucleoproteins.[a]

Source	CHR[b]	NPLS	NP 0.6	NP 1.0	NP 1.2	NP 3.0
Chick embryo brain	2.0–2.3	0.8–1.1	0.8–1.0	1.0–1.1	0.7–0.8	0.3
Pig cerebellum	1.8–2.1	1.3–1.6	1.1–1.3	1.2–1.4	1.0–1.1	0.8
Myeloma	1.0–1.3	0.6–0.9				0.2
Calf thymus	0.3–0.6		0.4–0.6	0.6	0.6	0.3
Chicken erythrocyte	0.9–1.2		0.7–0.9			0.2

[a] Calculated from the data in Tables I and II assuming that the histone/DNA ratios of the chromatins are 1.0–1.3 (Bonner *et al.*, 1968b). [b] Abbreviations are as for Tables I and II.

total protein to DNA mass ratio (Henson and Walker, 1970a) are consistent with the relationship observed here and in earlier work (Wilhelm *et al.*, 1970; Henson and Walker, 1970b) between the total protein to DNA mass ratios and the ellipticities at 283 nm of the samples (Tables I and III) suggesting a direct relationship between the unfolding of the DNA and the relaxation of the secondary structure of chromatin DNA to DNA free in solution. Furthermore, X-ray diffraction data (Skidmore *et al.*, 1973) of dissociated nucleohistone show the reflections characteristic of ordered chromatin in addition to reflections from DNA, even when all histones but the arginine-rich histone IV are removed.

Table V gives the estimated content of nonhistone protein in the various chromatins and depleted nucleoproteins. The numbers are determined from the values given on Tables I and II assuming histone to DNA mass ratios in the chromatins to be 1.0–1.3 (Bonner *et al.*, 1968b). A large amount of nonhistone, roughly 50%, is removed at very low saline concentrations (0.2–0.35 M), as determined in our forthcoming paper (Hjelm and Huang, 1974). Little or no nonhistones are removed by solutions containing higher concentrations of NaCl. Only treatment with 3.0 M NaCl appears to remove a significantly greater amount of salt-extractable nonhistones. Thus, the removal of these proteins does not correlate with the increase in ellipticity observed in samples treated with saline solutions. In fact, the relatively larger amounts of nonhistone removed by the higher concentration NaCl solutions account for the convex shape of curves in the graphs of ellipticity *vs.* protein to DNA mass ratio (Wilhelm *et al.*, 1970; Henson and Walker, 1970b). This indicates that the high salt-extractable nonhistones do not significantly affect the bulk properties of the chromatin DNA, at least as reflected by the CD. This is an extension of a similar observation on the low salt-extractable nonhistones in the related paper (Hjelm and Huang, 1974). A significant amount of residual nonhistone proteins remains on the DNA after dissociation with 3.0 M NaCl, yet we have observed that the CD of the 3.0 M NaCl depleted nucleoprotein is indistinguishable from that of DNA; thus, the residual nonhistone proteins also do not appear to affect the bulk CD properties of chromatin DNA. This conclusion is consistent with other observations on the CD of histone depleted nucleoprotein (Henson and Walker, 1970b; Simpson and Sober, 1970; Wilhelm *et al.*, 1970; Wagner and Spelsberg, 1971), and observations of the hydrodynamic (Henson and Walker, 1970a) and ethidium bromide binding properties (Angerer and Moudrianakis, 1972). This is in interesting contrast to the observed effect of the residual nonhistone proteins on the binding of actinomycin D to chromatin DNA (Kleiman and Huang, 1971).

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