

# Study of Conformational States and Reversibility of Histone Complexes<sup>†</sup>

Norman V. Beaudette,<sup>‡</sup> Andrew W. Fulmer,<sup>§</sup> Hirofumi Okabayashi, and Gerald D. Fasman<sup>\*,\*</sup>

**ABSTRACT:** The core histone complex (H3:H4:H2A:H2B)<sub>2</sub> and products of dissociation, the H2A:H2B dimer and the H3:H4 tetramer, were isolated from chicken erythrocyte chromatin by several literature methods as well as gel filtration on Bio-Gel A15m at various salt concentrations. The conformational and oligomeric characteristics of these histone complexes were compared to analogous histone complexes prepared by renaturation of individually acid-extracted histones by circular dichroism (CD) and analytical gel filtration chromatography. The salt-extracted core histone complex (independent of method of preparation), the purified dissociation products, the H2A:H2B dimer, and the H3:H4 tetramer in 2 M NaCl, 10

mM sodium phosphate, 0.25 mM EDTA, and 0.1 mM DTT, pH 7.0, have conformations which are identical, by the criteria of similar CD spectra, with complexes prepared from acid-extracted histones. Likewise, the salt-extracted complexes may be cycled through solvents of low ionic strength (10 mM sodium phosphate, pH 7.0, or 50 mM NaOAc, pH 5.0) or 1 mM HCl and returned to 2.0 M NaCl, 10 mM sodium phosphate, 0.25 mM EDTA, and 0.1 mM DTT, pH 7.0, in a completely reversible manner. Thus it would appear that acid-denatured histones are capable of being fully renatured to yield native-like complexes.

**E**ukaryotic interphase chromatin consists of a periodic array of histone complexes along the DNA. The basic repeating unit, known as the nucleosome, is composed of about 200 base pairs of DNA wrapped around a protein octamer consisting of two each of four core histones, H2A, H2B, H3, and H4, and one or two molecules of lysine-rich histone H1 (or H5 from avian erythrocytes) (Kornberg, 1974; Noll, 1974; Olins et al., 1976; Finch et al., 1977). Excellent reviews have been published by Kornberg (1977), Felsenfeld (1978), and McGhee & Felsenfeld (1980).

Considerable attention has been given to structural studies of histones and various histone complexes [for review, see Isenberg (1979)]. These studies serve as a pathway not only toward the complete understanding of chromatin structure but also toward an understanding of structural changes occurring in vivo, during such cellular processes as transcription and DNA replication.

Purification of individual core histone fractions requires denaturing solvent conditions, e.g., pH ~1.0 (Murray et al., 1968), high concentrations of denaturants such as urea (Van der Westhuyzen et al., 1974) and guanidine hydrochloride (Fambrough et al., 1968), and organic solvents (Oliver et al., 1972). The ability of denatured histones to renature to a native-like structure with the functional properties of histone complexes in chromatin has been questioned (Kornberg, 1977). Evidence for irreversible denaturation of histone complexes has been reported by Roark et al. (1976), Thomas & Kornberg (1975), Thomas & Butler (1977), and Mihalakis et al. (1976), while claims of reversibility have been made by Moss et al. (1976b), Ruiz-Carrillo & Jorcano (1979), and Bidney & Reeck (1977). There is still some controversy over this matter.

The most valid criterion for the native state of histone complexes is the demonstration of biological functionality, by condensation of high molecular weight DNA into nucleoprotein with the structural and functional properties of native chromatin. Reconstitution of high molecular weight chromatin using acid-extracted histones resulted in the appearance of nucleosomes in electron micrographs (Oudet et al., 1975), characteristic X-ray diffraction maxima (Boseley et al., 1976), and limit nuclease digestion products (Camerini-Otero et al., 1976). However, Garel et al. (1976) demonstrated that reconstitution with acid-extracted histones results in a mixture of well-organized nucleosomal subunits and nonspecific histone-DNA complexes. Both the specificity and ionic strength dependence of sequential histone complex binding to DNA were found to be different for acid-extracted histones and salt-dissociated complexes (Wilhelm et al., 1978). Reconstitution studies by Leffak & Li (1977), using acid-extracted histones and a variety of reconstitution pathways, have shown that neither a mixture of all five histones nor a mixture of the four core histones is capable of reassembling high molecular weight DNA into native chromatin-like material at physiological histone/DNA ratios as judged by circular dichroism and thermal denaturation. Such studies suggest that the structural integrity of acid-extracted histones is not completely regained upon "renaturation", under the conditions used.

We report studies herein on the conformational states of the histone octamer and products of dissociation, the H3:H4 tetramer and H2A:H2B dimer, obtained both by various relatively nondenaturing salt extraction procedures and by renaturation of mixtures of purified acid-extracted histones, in an attempt to rationalize the various conflicting published results. Conformational properties were compared by circular dichroism (CD).<sup>1</sup> Oligomeric properties were probed by analytical gel filtration chromatography. By these criteria,

<sup>†</sup> From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254. Received March 18, 1981. This is Publication No. 1382 from the Graduate Department of Biochemistry, Brandeis University. This research was generously supported in part by grants from the U.S. Public Health Service (GM 17533), the American Cancer Society (P-577), and the U.S. Department of Energy (EP-78-S-02-4962.A000). Submitted in part by A.W.F. in partial fulfillment of the requirements for the Ph.D. degree at Brandeis University.

<sup>‡</sup> N.V.B. was supported by a Postdoctoral Fellowship of the American Cancer Society (PF-1592).

<sup>§</sup> A.W.F. was a predoctoral trainee on Training Grant 5701 GM 00212 from the National Institutes of Health.

<sup>\*</sup> G.D.F. is the Rosenfield Professor of Biochemistry.

<sup>1</sup> Abbreviations used: CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; NaDodSO<sub>4</sub>, sodium dodecyl sulfate; NaOAc, sodium acetate; Tris buffer, 10 mM Tris and 0.25 mM EDTA, pH 8.0; 2 M NaCl, pH 7 buffer, 2 M NaCl, 10 mM sodium phosphate, and 0.25 mM EDTA, pH 7.0; pH 7 phosphate buffer, 10 mM sodium phosphate and 0.25 mM EDTA, pH 7.0; pH 5 acetate buffer, 50 mM NaOAc and 0.25 mM EDTA, pH 5.0; PMSF, phenylmethanesulfonyl fluoride.

all the salt-extracted histone complexes examined appear identical with the corresponding renatured histone complexes. These studies indicate that the native state of the core histone complex (isolated in 2 M NaCl) or its dissociation products, H2A:H2B dimer and the H3:H4 tetramer, can be simulated by the use of acid-denatured histones. Thus conformational changes in the core complex, H2A:H2B dimer, and H3:H4 tetramer are completely reversible, if sufficient care is taken in these operations. A new procedure for salt dissociation of histones from agarose gels is reported to obtain the three species listed above.

### Materials and Methods

**Isolation of Chromatin.** Chicken erythrocyte nuclei were prepared by the method of Ramsay-Shaw et al. (1976). High molecular weight chromatin was solubilized by digestion with micrococcal nuclease at 0 or 37 °C and extracted with EDTA, as described by Fulmer & Fasman (1979). All subsequent histone purification procedures were performed at 0–5 °C.

**Acid Extraction of Histones.** Histones were extracted from chicken erythrocyte chromatin with 3.5 N HCl by the method of Murray et al. (1968) as described by Nicola et al. (1978). Isolation of individual core histone fractions was achieved on Bio-Gel P-60 (Van der Westhuyzen et al., 1974) as described by Nicola et al. (1978). The purified histones were exhaustively dialyzed against  $10^{-3}$  N HCl, lyophilized, and stored in a desiccator at –20 °C.

**Salt Extraction of Histone Complexes.** (a) *Agarose Method.* Salt-extracted histone complexes were isolated by stepwise salt dissociation (Ohlenbusch et al., 1967) of specific histone fractions from high molecular weight chromatin.

(i) *Removal of Low Molecular Weight Nucleoprotein.* Approximately 70 mg of whole chromatin was fractionated by gel filtration by using a  $2.0 \times 70.0$  cm column packed with Bio-Gel A15m (Bio-Rad 100–200 mesh) equilibrated with 10 mM Tris-HCl and 0.25 mM EDTA, pH 8.0 (Tris buffer), at 4 °C. About 50 mg of high molecular weight chromatin eluted in the void volume and was subsequently pooled and concentrated by ultrafiltration over PM-10 membranes (Amicon Corp.) operated at 30 psi of  $N_2$ .

(ii) *Removal of Lysine-Rich Histones. Core Chromatin.* Approximately 50 mg of concentrated chromatin (~2 mg/mL) was dialyzed for 24 h in Spectrapore 3 dialysis tubing (treated as described below) against the above buffer containing 0.6 M NaCl. Fractionation proceeded on an identical gel filtration column as above, equilibrated with the 0.6 M NaCl-Tris buffer. DNA and associated core histones (core chromatin) eluted in the void volume (Figure 1A) while lysine-rich histones, H1 and H5, eluted in a peak at higher elution volumes. Core chromatin fractions were pooled as indicated (horizontal bars) and reduced to about 20 mL by ultrafiltration, as described above. Concentrations of whole and core chromatin were calculated by using protein to DNA weight ratios of 1.05 and 0.79, respectively (Fulmer & Fasman, 1979). DNA concentration was calculated from absorbance at 260 nm, using  $OD_{260}^{1\text{cm}} = 20$  for 1 mg/mL.

(iii) *Isolation of Salt-Extracted H2A:H2B Complex.* The concentrated core chromatin was next dialyzed for 24 h against Tris buffer containing 1.25 M NaCl followed by fractionation on a third identical Bio-Gel column equilibrated with the 1.25 M NaCl-Tris buffer. This resulted in the separation of the H3:H4-DNA nucleoprotein complex, which elutes in the void volume, from H2A:H2B, which elutes at higher volumes (Figure 1B). Fractions containing H2A:H2B were pooled as indicated in Figure 1B, concentrated to about 1 mg/mL by vacuum dialysis employing collodion bags (Schleicher &

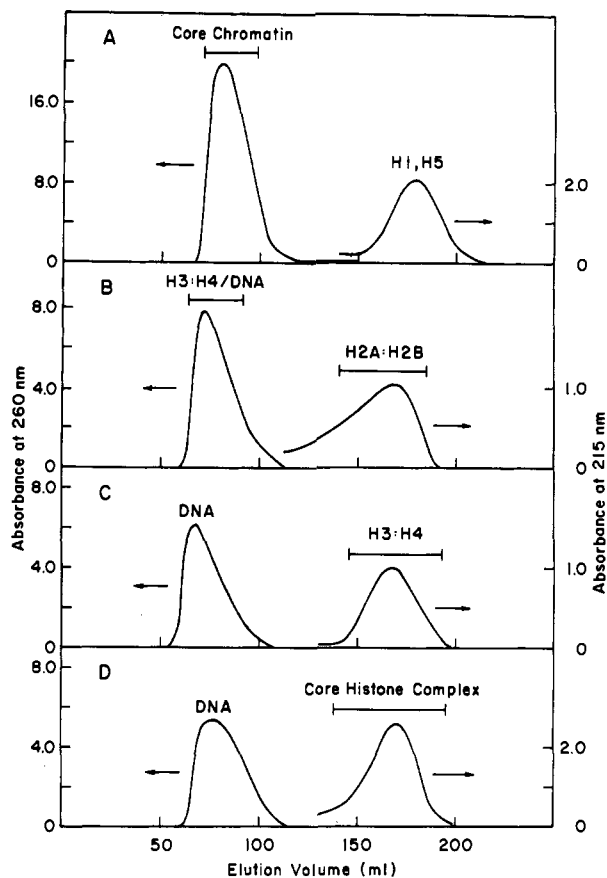


FIGURE 1: Salt extraction of histone complexes by gel filtration on a  $2 \times 70$  cm column of Bio-Gel A-15m. All buffers contained 10 mM Tris-HCl, 0.25 mM EDTA, and NaCl at the concentration indicated below. The pH of each buffer was adjusted to pH 8.0. Fractions of 5 mL were collected at 4 °C at a rate of 20 mL/h. Fractions were pooled as indicated by the bars. Absorbance is reported for a 1.0-cm path length. (a) Purification of core chromatin by dissociation of H1 and H5 from whole chromatin in 0.6 M NaCl. (b) Purification of H2A:H2B from core chromatin by dissociation in 1.25 M NaCl. (c) Purification of H3:H4 from H3:H4-DNA by dissociation in 2.0 M NaCl. (d) Purification of core histone complex from core chromatin by dissociation in 2.0 M NaCl.

Schuell, 10 000-dalton cutoff), followed by exhaustive dialysis against 2.0 M NaCl, 10.0 mM sodium phosphate, and 0.25 mM EDTA, pH 7.0 (2 M NaCl, pH 7 phosphate buffer), and stored at 4 °C or frozen at –20 °C.

(iv) *Isolation of Salt-Extracted H3:H4 Complex.* Fractions containing the H3:H4-DNA complex were pooled as indicated in Figure 1B, reduced in volume to ~20 mL by ultrafiltration, as described above, and dialyzed for 24 h against 2.0 M NaCl-Tris buffer. Fractionation on a fourth Bio-Gel column, of identical dimensions, equilibrated with the same 2.0 M NaCl-Tris buffer resulted in the separation of DNA, eluting in the void volume, from H3:H4, eluting at higher volumes as shown in Figure 1C. Fractions containing the H3:H4 complex were pooled as indicated, concentrated to ~1 mg/mL by vacuum dialysis, followed by exhaustive dialysis against 2.0 M NaCl, pH 7 phosphate buffer, and stored at 4 °C or frozen at –20 °C.

(v) *Isolation of Core Histone Complex.* The core histone complex containing all four histones in equimolar amounts was isolated by the same procedure, but excluding the 1.25 M NaCl-Tris gel filtration step. Concentrated core chromatin was dialyzed directly against 2.0 M NaCl-Tris buffer for 24 h before fractionation on the final column. This resulted in two well-resolved peaks (Figure 1D). DNA eluted in the void volume, and the four core histones in approximately equimolar

ratios eluted in the higher volume. Core fractions were pooled as indicated in Figure 1D, concentrated by vacuum dialysis, dialyzed against 2.0 M NaCl, pH 7 phosphate buffer, and stored at 4 °C or frozen at -20 °C.

It is important to use only nucleoprotein which elutes in the void volume peaks of the above columns in each step of the purification. Flow rates of all column fractionations were approximately 20 mL/h. Fractions of 5 mL were collected. The concentrations of DNA and protein were monitored by absorption at 260 and 215 nm, respectively. Less than 50 mg of nucleoprotein in 20–25 mL was applied to each of the columns. Yields of purified histone complexes were approximately 50%. Most of the losses could be attributed to the steps of nucleoprotein concentration and could be minimized somewhat by using fresh PM-10 membranes each time and by allowing the concentrated nucleoprotein solution to stir in the ultrafiltration apparatus for about 20 min after the pressure was released.

**(b) Hydroxylapatite Method. H2A:H2B and H3:H4 Complexes.** Histone pairs were isolated from chromatin (prepared as above) on hydroxylapatite by the method of Simon & Felsenfeld (1979) with the following modifications. Potassium salts were used in place of sodium salts to overcome crystallization of buffer salts at high concentration (L. Feldman and B. D. Stollar, personal communication). The chromatin was loaded on a hydroxylapatite column, which had been preequilibrated in buffer in which the chromatin is applied (34 mg of DNA in ~20 mL of 0.63 M KCl, 0.10 M potassium phosphate, and 0.10 mM DTT, pH 6.8). The hydroxylapatite column (2.5 × 20 cm, Bio-Gel HTP) was washed with 600 mL of the same buffer. The column was then washed with 350 mL of 0.93 M KCl, 0.1 M potassium phosphate, and 0.1 mM DTT, pH 6.8. Next a 500-mL linear gradient from 0.93 to 1.20 M KCl was applied; after the salt gradient, an additional 200 mL of the 1.2 M KCl, 0.10 M potassium phosphate, and 0.1 mM DTT, pH 6.8, was passed through the column; a final 200 mL of 2 M KCl, 0.10 M potassium phosphate, and 0.1 mM DTT, pH 6.8, was used to elute the H3:H4. Analysis of column fractions by acid-urea-polyacrylamide gel electrophoresis (Panyim & Chalkley, 1969) revealed that the leading edge of the H2A:H2B peak contained considerable contaminating H5. These fractions were rejected, and only those fractions containing H5-free H2A:H2B were pooled and concentrated by using Amicon PM-10 ultrafiltration membranes. Histone pairs were dialyzed into 2.0 M NaCl, pH 7 phosphate buffer with 0.1 mM DTT added and stored at -20 °C in 2-mL Nunc tubes (InterMed Denmark).

**(c) Combined Agarose-Hydroxylapatite Method. (i) H2A:H2B and H3:H4 Complexes.** The following is a modification of the hydroxylapatite method in which H5 contamination is eliminated by removal of H5 prior to hydroxylapatite chromatography. Whole chromatin was stripped of H1 and H5 by overnight dialysis into 0.6 M NaCl, 0.01 M Tris, 0.25 mM EDTA, 0.10 mM DTT, and 0.10 mM PMSF, pH 8.0, followed by fractionation on a 2.5 × 110 cm Bio-Gel A15m gel filtration column (Bio-Rad, 100–200 mesh) equilibrated with the same buffer. On this column, H1 and H5 are transported very slowly relative to nucleoprotein (see Figure 1A) and are readily distinguished by their higher ratio of 215–260-nm absorbance. Fractions of 12.5 mL were collected at an average flow rate of approximately 20 mL/h. The core chromatin obtained by this method was next diluted with one part 0.6 M sodium phosphate, pH 6.7, to five parts core chromatin to make the chromatin solution 0.5 M in NaCl and 0.10 M in phosphate, pH 6.7. A volume of 126 mL of this

material (0.32 mg DNA/mL) was absorbed on a 2.5 × 20 cm column of hydroxylapatite HTP (Bio-Rad) which had been preequilibrated with 0.5 M KCl, 0.10 M potassium phosphate, and 0.10 mM DTT, pH 6.7. The column was then washed with an additional 450 mL of the same preequilibration buffer.

H2A:H2B and H3:H4 were removed from the hydroxylapatite-bound DNA by consecutive washings with 500 mL of 1.0 M KCl and 500 mL of 2.0 M KCl, both buffers containing 0.10 M potassium phosphate and 0.10 mM DTT, pH 6.7. Fractions of 8 mL were collected at an average flow rate of 20–30 mL/h. Peak fractions were pooled, concentrated by ultrafiltration using PM-10 membranes, dialyzed for 48 h into 2.0 M NaCl, pH 7 phosphate buffer with 0.10 mM DTT, and stored in plastic Nunc tubes at -20 °C. The hydroxylapatite column was washed and stripped of bound DNA with 500 mL of 0.5 M potassium phosphate, pH 6.7, and then restored with the 0.5 M KCl, phosphate starting buffer.

**(ii) Core Histone Complex.** Core complexes were prepared by using agarose and hydroxylapatite by a modification of the method of Rhodes (1979). Whole chromatin was obtained as above. H1 and H5 were stripped from core chromatin on a Bio-Gel A15m column in 0.6 M NaCl-Tris buffer, as described above. The core chromatin was then concentrated by ultrafiltration (PM-10 membranes) to about 2 mg/mL and dialyzed 12 h against 2.0 M KCl, 0.1 M potassium phosphate, and 0.1 mM PMSF, pH 6.7. Fractionation on a hydroxylapatite column (1.6 × 15 cm) then proceeded as described by Rhodes (1979). Core histone fractions were pooled, concentrated by ultrafiltration (PM-10 membranes) to about 1 mg/mL, centrifuged at 12000g for 30 min to remove trace aggregates, and stored at -20 °C.

**(d) Sucrose Gradient Method. Core Histone Complex.** The core complex was also isolated by using the method of Butler et al. (1979). Chicken erythrocyte nuclei were prepared by the method of Ramsay-Shaw et al. (1976), extracted with 0.35 M NaCl and 1.0 mM PMSF, swollen in 1.0 mM EDTA, and lysed in glass-distilled water. This crude chromatin was made 10 mM in Tris (pH 7.4), 10 mM in DTT, and 0.1 mM in PMSF. Solid NaCl was added to a final concentration of 2 M. DNA was removed by centrifugation at 48 000 rpm for 12 h at 4 °C (Beckman L-2, SV-50 head). The supernatant was collected and concentrated by vacuum dialysis. The concentrated NaCl extract was layered on 5–20% sucrose gradients and centrifuged for 44 h. The inner histones (core was separated from the H1 and H5 and collected separately) were dialyzed against 2.0 M NaCl, 10.0 mM sodium phosphate, 0.25 mM EDTA, 0.1 mM DTT, and 0.1 mM PMSF, pH 7.2, concentrated to about 2 mg/mL by vacuum dialysis, centrifuged for 30 min at 12000g to remove aggregates, and stored at -20 °C in plastic 2-mL Nunc tubes (InterMed Denmark).

**Renaturation of Acid-Extracted Histones into Complexes.** Solutions of acid-extracted core histones were prepared by dissolving lyophilized histone powders in 10<sup>-3</sup> N HCl to concentrations of ~5 mg/mL. Solid urea was added to a final concentration of ~8 M estimated from a volume increase of 1.5-fold. Tris-HCl, pH 8.8, and DTT were added to final concentrations of 0.1 M and 5 mg/mL, respectively, from a 10-fold concentrated stock solution. Solutions were incubated at 40 °C for 1 h before exhaustive dialysis against 10<sup>-3</sup> N HCl at 4 °C. This preparation results in clear solutions displaying negligible light scattering from 400 to 300 nm. Concentrations of histones in solutions of 10<sup>-3</sup> N HCl were determined by the following molar extinction coefficients at 275 nm: H3, 5.5 × 10<sup>3</sup> cm<sup>-1</sup> M<sup>-1</sup>; H4, 5.3 × 10<sup>3</sup> cm<sup>-1</sup> M<sup>-1</sup>; H2A, 4.0 × 10<sup>3</sup> cm<sup>-1</sup>

M<sup>-1</sup>; H2B,  $6.1 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1}$ . The extinction coefficients were calculated from the absorption spectra and the total nitrogen concentration determined by the Kjeldahl method described by Jones (1960) and using the amino acid compositions reported by Olins & Wright (1973). Renatured histone complexes of H3:H4 and H2A:H2B were prepared by mixing the appropriate stock histone solutions at 1:1 molar ratios followed by denaturation and reduction with urea and DTT as described above. Denaturants were removed by dialysis against  $2 \times 1 \text{ L}$  of  $10^{-3} \text{ N HCl}$  for 48 h at 4 °C. Renaturation was achieved by dialysis against 1 L of 2.0 M NaCl, 10.0 mM sodium phosphate, and 0.25 mM EDTA, pH 7.0, at 4 °C for 24 h. Approximately 2–4 mg of renatured histone complex in ~2 mL was fractionated on a  $1.2 \times 50 \text{ cm}$  gel filtration column packed with Bio-Gel A0.5m equilibrated with 2.0 M NaCl, pH 7 phosphate buffer at a flow rate of ~8 mL/h at 4 °C. This fractionation is required to remove irreversibly formed histone polymers from the heterogeneous mixture of renatured histone species [see Nicola et al. (1978)]. Renatured H3:H4 tetramer and H2A:H2B dimer were pooled and concentrated to ~1 mg/mL by vacuum dialysis.

**Cycling of Salt-Extracted Histone Complexes.** Salt-extracted histone complexes were dialyzed from 2 M NaCl, pH 7 phosphate buffer to 10 mM sodium phosphate and 0.25 mM EDTA, pH 7.0, or 50 mM sodium acetate and 0.25 mM EDTA, pH 5.0, or  $10^{-3} \text{ N HCl}$  for 2–3 days. These solutions were then dialyzed back to the original 2 M NaCl, pH 7 phosphate buffer for at least 24 h. Those histones which had been dialyzed against HCl were restored to the high-salt buffer through an intermediate dialysis against 10 mM sodium phosphate, 0.25 mM EDTA, and 0.10 mM DTT, pH 7.0, for an additional 24 h.

**Spectroscopic Analysis of Histone Complexes.** Absorption spectra were recorded on a Cary 14 spectrophotometer in 1.0-cm Suprasil cells (Hellma Scientific, 1–160 BQS) at 4 °C. Circular dichroism (CD) measurements were made with a Cary 60 recording spectropolarimeter equipped with a Model 6001 CD accessory, as previously described (Alder et al., 1971), with the original photomultiplier tube replaced with an end-on Hamamatsu tube (R375). All measurements were made at  $5 \pm 1$  °C in jacketed 0.1–1.0-mm path length cells (Optical Cell Co.) at full-scale sensitivities of 0.04 or 0.02°. Ellipticity,  $[\theta]$ , is expressed in degree centimeter squared per decimole of amino acid residue. Reported spectra represent averages of two to three independent preparations. The molecular weights used for the H3:H4 dimer, H2A:H2B, and average amino acid residue were 26 600, 28 200, and 111, respectively. Concentrations of histone complexes were determined by the method of Lowry et al. (1951) with purified histone H4 as the protein standard. In practice, BSA (Bio-Rad Standard) was used for the calibration curves, and each batch of BSA was carefully calibrated against the purified H4.

**Gel Electrophoresis of Histones.** Purity of individual histones and histone complexes was assessed by gel electrophoresis on 20% polyacrylamide–acetic acid–urea gels (Panyim & Chalkley, 1969). Gels were stained with 0.1% Amido Black in methanol/acetic acid/water (5:1:5), diffusion destained in the same solvent, and scanned at 570 nm with a Zeiss spectrophotometer equipped with a linear transport device. Areas of the stained histone bands were determined with a Du Pont 310 curve resolver. The ratios of the histones in the various complexes are shown in Table I. The absence of nonhistone proteins in these preparations was determined by the sodium dodecyl sulfate–polyacrylamide gel electrophoresis method of Maizel (1971) as described by Fulmer & Fasman (1979).

Table I: Ratios of Histone Components Isolated by Various Methods

	H3	H2B	H2A	H4
Core Histone Complex				
salt extracted				
agarose	1.22	2.10	1.22	1 <sup>a</sup>
sucrose gradient	1.30	1.90	0.90	1
hydroxylapatite	1.40	2.30	1.20	1
renatured	1.35	2.20	1.40	1
H3:H4				
salt extracted				
agarose	0.96			1 <sup>b</sup>
hydroxylapatite	1.4			1
renatured	1.0			1
H2A:H2B				
salt extracted				
agarose		2.1	1	
hydroxylapatite		2.2	1	
renatured		2.3	1	

<sup>a</sup> The numbers represent the percent of the total histone band areas, on acetic acid–urea–polyacrylamide gels stained with Amido Black, for each band and divided by the H4 area. Averages of three to four determinations were reproduced to  $\pm 5\%$  of the total area. <sup>b</sup> As in footnote <sup>a</sup>, but areas divided by the H2A area.

**Miscellaneous.** All dialyses were performed with Spectrapor 3 membrane tubing (Spectrum Medical Industries, Inc.). Dry membrane tubing was suspended in 10 mM NaHCO<sub>3</sub> and 1 mM EDTA and heated on a steam bath for ~0.5 h. Extensive rinsing with glass-distilled water was followed by heating the tubing in glass-distilled water on a steam bath for an additional ~0.5 h. The tubing was then extensively rinsed with glass-distilled water and stored wet at 4 °C. A Radiometer Model 25 pH meter with a Radiometer Type GK 2302 combination electrode was used to measure the pH.

**Proteins Used.** The following proteins were used for calibration of gel filtration columns: D-amino acid oxidase, Worthington, lot DAO 623; bovine serum albumin, Pentex, Inc., lot 9; Ovalbumin, Mann Research Labs, lot 7338; DNase I, Worthington, lot D57C377;  $\alpha$ -chymotrypsin, Worthington, lot CD1 6084-5.

All chemicals were reagent grade. Solutions were prepared at room temperature with glass-distilled water.

## Results

**H3:H4.** The H3:H4 complexes isolated by the agarose or hydroxylapatite methods, or by renaturation of acid-extracted H3 and H4, contained no detectable nonhistone proteins and were at least 97% pure as determined by acid–urea–polyacrylamide gel electrophoresis (results not shown). H3:H4 isolated by the combined agarose–hydroxylapatite method was at least 95% pure; H2A and H2B were the principle contaminants. The latter method has the advantages of being relatively fast and efficient (~80% recovery of H3:H4). The relative staining intensities of H3 to H4 obtained in all preparations were identical within experimental error (Table I). Thus, the arginine-rich histone complexes all appear to be identical with respect to protein composition.

A comparison of the oligomeric properties of salt-extracted H3:H4 obtained by the agarose method, with acid-renatured H3:H4 in 2 M NaCl, pH 7 phosphate buffer, was made by gel filtration chromatography (Figure 2a). No aggregated material was detected in the void volume of either salt-extracted or previously fractionated renatured H3:H4 [see Nicola et al. (1978)]. The peak position and elution half-widths are essentially identical for the two complexes. They migrate with

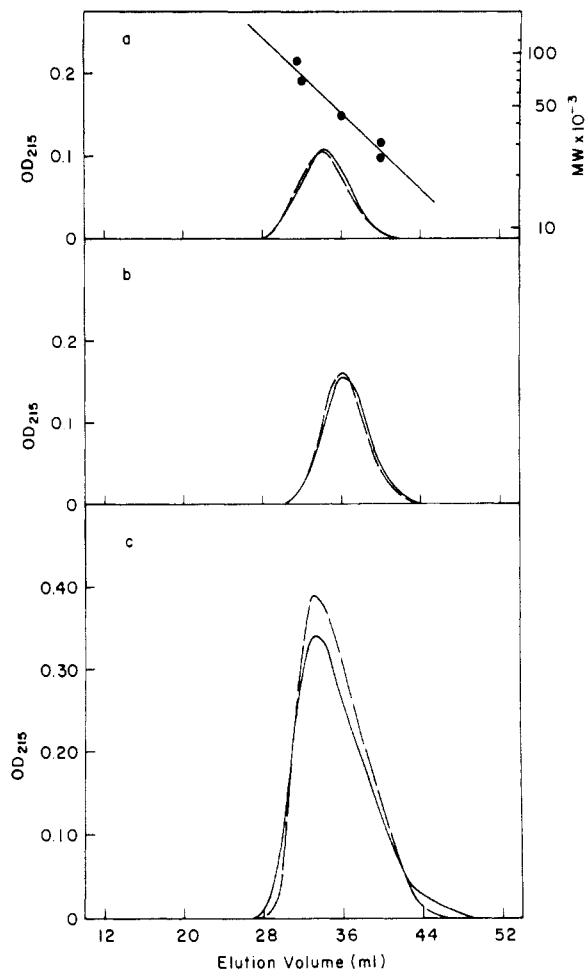


FIGURE 2: Comparison of salt-extracted and acid-renatured histone complexes by analytical gel filtration. A  $1.2 \times 50$  cm column of Bio-Gel A0.5m was equilibrated with 2.0 M NaCl, 10.0 mM sodium phosphate, and 0.25 mM EDTA, pH 7.0, at 4 °C and processed at 8 mL/h. The column was calibrated with globular proteins of known molecular weight: D-amino acid oxidase,  $M_r$  90 000; bovine serum albumin,  $M_r$  67 500; ovalbumin,  $M_r$  44 000; DNase I,  $M_r$  31 000;  $\alpha$ -chymotrypsin,  $M_r$  25 000. Each protein was dissolved in the equilibrating buffer at 2 mg/mL, and approximately 0.3 mg of each was independently fractionated. (a) Calibration curve and elution profiles for 0.097 mg of salt extracted H3:H4 (—) and 0.097 mg of renatured H3:H4 (---). (b) Elution profiles for 0.103 mg of salt-extracted H2A:H2B (—) and 0.103 mg of renatured H2A:H2B (---). (c) Elution profiles for 0.316 mg of salt-extracted core histone complex (—) and 0.323 mg of renatured core histone complex (---).

an apparent molecular weight of 55 000, as determined by calibration with the globular protein standards described in the legend to Figure 2. This is consistent with a multiple chemical equilibrium between monomers, dimers, and tetramers of H3 and H4 (Roark et al., 1974, 1976; D'Anna & Isenberg, 1974b).

The peptide circular dichroism (CD) spectrum of renatured H3:H4 in 2 M NaCl, 10 mM sodium phosphate, and 0.25 mM EDTA, pH 7, is shown in Figure 3. Ellipticity extrema are observed at 208 ( $[\theta]_{208} = -17\,200$  deg cm<sup>2</sup>/dmol) and 222 nm ( $[\theta]_{222} = -14\,800$  deg cm<sup>2</sup>/dmol). The peptide CD spectra of H3:H4 isolated by the agarose, hydroxylapatite, and combined procedures are also given in Figure 3. The differences among all four spectra are well within the combined experimental errors of spectrum measurement, determination of protein concentrations, and pipetting. Characterization of the protein secondary structure is possible by CD analysis in the peptide chromophore absorption region, 195–250 nm (Greenfield & Fasman, 1969; Chen et al., 1972). Computer estimation of secondary structure from the CD spectra yielded

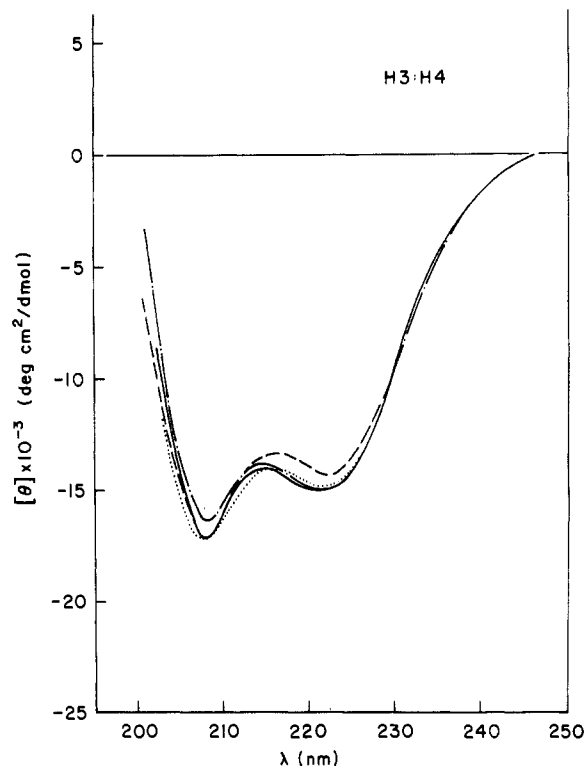


FIGURE 3: Circular dichroism spectra of the peptide region of H3:H4 in 2.0 M NaCl, 10.0 mM sodium phosphate, and 0.25 mM EDTA, pH 7.0, at 4 °C. All spectra were recorded in 0.2- or 0.5-mm jacketed cells at protein concentrations of 1.0–1.7 mg/mL. Methods of isolation: (a) agarose filtration (---); (b) hydroxylapatite (---); (c) agarose and hydroxylapatite (—); (d) acid renaturation (---).

an average of 35%  $\alpha$ -helical content and 7%  $\beta$ -sheet content on the basis of Greenfield & Fasman (1969) method. Differences in estimates are obtained by the two methods (see Table II for summary).

**H2A:H2B.** Renatured H2A:H2B and all salt-extracted H2A:H2B complexes contained no detectable nonhistone proteins and were at least 97% pure with respect to other histones, as determined by polyacrylamide gel electrophoresis (results not shown). The relative staining intensities of H2B to H2A were identical within experimental error for all H2A:H2B preparations (Table I).

The oligomeric properties of salt-extracted H2A:H2B obtained by the agarose method were compared to renatured H2A:H2B in 2 M NaCl, pH 7 phosphate buffer by gel filtration chromatography (Figure 2b). No aggregated material was detected in the void volume of either salt-extracted or previously fractionated small molecular weight renatured H2A:H2B [see Nicola et al. (1978)]. The peak position and elution half-widths are essentially identical for the two complexes. They migrate with an apparent molecular weight of  $\sim 42\,000$ , as determined by the globular protein standards described in the legend to Figure 2. This is consistent with a multiple chemical equilibrium between somewhat asymmetric monomers and dimers of H2A and H2B (Roark et al., 1976; Kelley, 1973; D'Anna & Isenberg, 1974c).

Spectral characterization of the H2A:H2B secondary structure by CD is shown in Figure 4. Renatured H2A:H2B in 2 M NaCl, 10 mM sodium phosphate, and 0.25 mM EDTA, pH 7, has ellipticity extrema at 208 ( $[\theta]_{208} = -18\,200$  deg cm<sup>2</sup>/dmol) and 222 nm ( $[\theta]_{222} = -17\,200$  deg cm<sup>2</sup>/dmol). The peak ellipticities of H2A:H2B isolated by all four procedures are all similar within experimental error (Figure 4). Computer estimation of secondary structure from the CD spectra yielded 44%  $\alpha$ -helical content and 7%  $\beta$ -sheet content

Table II: Estimates of Secondary Structure in Histone Complexes by Analysis of Peptide CD Spectra<sup>a</sup>

histone complex	solvent <sup>g</sup>	$[\theta]_{208}^h$ (deg cm <sup>2</sup> /dmol)	$[\theta]_{222}$ (deg cm <sup>2</sup> /dmol)	PLL <sup>b</sup>			proteins <sup>c</sup>		
				$\alpha$ (%)	$\beta$ (%)	$\pm[\theta]^d$	$\alpha$ (%)	$\beta$ (%)	$\pm[\theta]^d$
H3:H4 <sup>e</sup>	I	-17 000 $\pm$ 900	-14 700 $\pm$ 700	35	7	687	44	4	2171
H2A:H2B <sup>e</sup>	I	-19 000 $\pm$ 1000	-17 400 $\pm$ 900	44	7	654	50	2	1166
core histone <sup>f</sup>	I	-18 600 $\pm$ 1000	-16 800 $\pm$ 750	40	8	581	47	7	1175
H3:H4	II	-22 500 $\pm$ 2000 (198)	-2 400 $\pm$ 500	0	0				
H2A:H2B	II	-25 700 $\pm$ 2000 (198)	-2 800 $\pm$ 500	0	0				
core histone	II	-21 000 $\pm$ 2000 (198)	-1 700 $\pm$ 500	0	0				
H3:H4	III	-13 900 $\pm$ 1000 (203)	-8 000 $\pm$ 500	13	3	968	18	13	2876
H2A:H2B	III	-15 500 $\pm$ 1000 (202)	-4 200 $\pm$ 500	0	7	958	5	14	2412
H3:H4	IV	-12 800 $\pm$ 800	-9 400 $\pm$ 500	17	18	1125	26	10	1193
H2A:H2B	IV	-17 000 $\pm$ 800	-14 400 $\pm$ 500	38	7	853	45	8	1925

<sup>a</sup> Percent of  $\alpha$  helix and  $\beta$  sheet were determined from the CD spectra from 201 to 250 nm. Ellipticities were compiled in  $\sim 2$ -nm increments and processed by computer with successive deletions of two data points from the low wavelength extrema for four cycles of curve fitting. The fractions derived from spectra displaying low values of  $\pm[\theta]$  were usually insensitive to the wavelength region used in the fitting procedure. The fractions derived from spectra displaying high values of  $\pm[\theta]$  were somewhat sensitive to the wavelength region used in the fitting procedure. The reported values for these spectra are the best estimates taken from the more insensitive regions of the wavelength window. <sup>b</sup> Using  $\alpha$ -helical and  $\beta$ -sheet CD reference spectra of poly(L-lysine) (Greenfield & Fasman, 1969) and random coil CD reference taken as the average of spectra of individual core histones in  $10^{-3}$  N HCl. <sup>c</sup> Using  $\alpha$ -helical,  $\beta$ -sheet, and random coil reference spectra derived from globular proteins by Chen et al. (1972). <sup>d</sup> Standard deviation between observed and calculated CD spectra from points for 2 nm from 201 to 250 nm in units of degree centimeter squared per decimole of amino acid residue. Spectra displaying  $\pm[\theta] < 1000$  deg cm<sup>2</sup>/dmol frequently result in calculated spectra which are contained within the order lines of experimental error of all wavelengths above 201 nm. Spectra displaying  $\pm[\theta] > 1000$  deg cm<sup>2</sup>/dmol result in less desirable fits, and the resulting secondary structure estimates are consequently less reliable. <sup>e</sup> Averaged values from histone preparations prepared by HCl acid extraction, agarose, hydroxylapatite, and agarose-hydroxylapatite. Concentration =  $\sim 1.25$  mg/mL. See Materials and Methods. <sup>f</sup> Averaged values from histone prepared by footnote e above plus sucrose gradient. Concentration =  $\sim 0.65$  mg/mL. See Materials and Methods. <sup>g</sup> Solvent systems: solvent I, 2.0 M NaCl, 10 mM sodium phosphate, 0.25 mM EDTA, and 0.1 mM DTT, pH 7.0; solvent II, 1 mM HCl; III, 50 mM NaOAc and 0.25 mM EDTA, pH 5.0; solvent IV, 10 mM sodium phosphate and 0.25 mM EDTA, pH 7.0. <sup>h</sup> Measured at 208 nm unless otherwise indicated in parentheses.

by the Greenfield & Fasman (1969) method.

**Core Histone Complex.** The core histone complex was prepared in five different ways: renaturation from acid-extracted histones, chromatography on agarose gel or on agarose followed by hydroxylapatite, sucrose gradient centrifugation, and by mixing histone pairs obtained by the combined agarose-hydroxylapatite method. All five procedures yielded pure complex, free of nonhistone proteins and lysine-rich histones, as detected by polyacrylamide gel electrophoresis (results not shown). Due to the higher propensity for extensive irreversible aggregation by mixtures of H3 and H4 over those of H2A and H2B [see Nicola et al. (1978)], because of the high concentrations used in these experiments (10 mg/mL), attempts to renature mixtures of the four core histones resulted in an enrichment of H2A:H2B over H3:H4. Thus, the stoichiometry of renatured core histone complexes was controlled by mixing the renatured histone pairs at equimolar ratios. The relative staining intensities of the core histones were identical within experimental error for salt-extracted and renatured core histones (Table I).

The oligomeric properties of the salt-extracted core histone complex were compared to renatured core histone complex in 2 M NaCl, pH 7 phosphate buffer by gel filtration chromatography as shown in Figure 2c. No aggregated material was detected in the void volume of either core histone mixture. The peak position and elution half-widths are essentially identical for the two complexes. They migrate with an apparent molecular weight of  $\approx 65$  000, as determined by the globular protein standards described in the legend to Figure 2. The peak is followed by a rather broad trailing boundary. This elution is consistent with a multiple chemical equilibrium between H3:H4 tetramers and H2A:H2B dimers with hexameric and octameric products of association (Eickbush & Moudrianakis, 1978; Ruiz-Carrillo & Jorcano, 1979).

The CD spectra of the core histone complexes examined are shown in Figure 5. Ellipticity extrema are observed for the renatured complex at 208 ( $[\theta]_{208} = -17$  600 deg cm<sup>2</sup>/dmol) and 222 nm ( $[\theta]_{222} = -15$  800 deg cm<sup>2</sup>/dmol). All the other

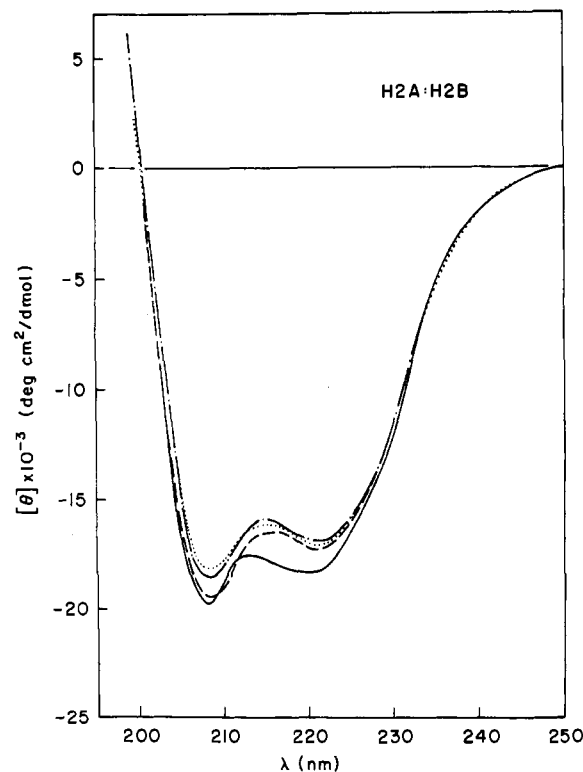


FIGURE 4: Circular dichroism spectra of the peptide region of H2A:H2B in 2.0 M NaCl, 10.0 mM sodium phosphate, and 0.25 mM EDTA, pH 7.0, at 4 °C. Methods of isolation and spectral analysis are the same as in the legend to Figure 3. Protein concentrations were from 0.3 to 1.2 mg/mL.

preparations examined have somewhat more negative ellipticities at the extrema. While differences between the spectra may be due primarily to experimental error, it has been found (H. Okabayashi, N. V. Beaudette, and G. D. Fasman, unpublished results) that at high protein concentration, core histone complex obtained under nondissociating conditions (2 M NaCl) differs in its CD spectrum from that obtained by

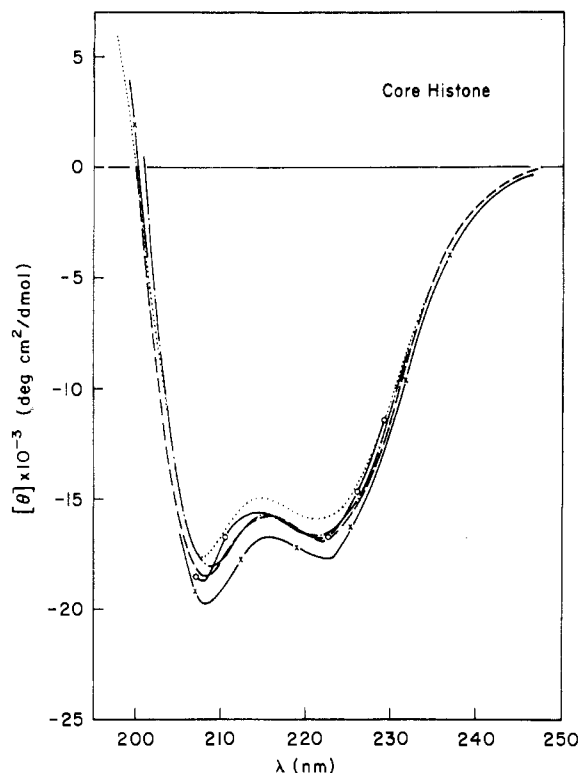


FIGURE 5: Circular dichroism spectra of the peptide region of core histone complex in 2.0 M NaCl, 10.0 mM sodium phosphate, and 0.25 mM EDTA, pH 7.0, at 4 °C. All spectra were recorded in 0.2- or 0.5-mm jacketed cells at protein concentrations of 0.3–1.0 mg/mL. Methods of isolation: (a) agarose filtration (—○—); (b) mixing of histone pairs prepared by agarose and hydroxylapatite (—·—·—); (c) prepared directly on agarose and hydroxylapatite (—×—); (d) sucrose gradient (---); (e) mixing of acid-renatured pairs (···).

mixing the appropriate pairs. Computer estimation of core particle averaged secondary structure yielded 40%  $\alpha$ -helical content and 8%  $\beta$ -sheet content (see summary Table II).

**Cycling Studies.** For reconciliation of reports of conformational identity between renatured and “native” H3:H4 complexes (Bidney & Reeck, 1977; Moss et al., 1976b), the peptide CD spectra of salt-extracted H3:H4 were examined under different solvent conditions, and reversibility was studied upon return to 2.0 M NaCl, pH 7 phosphate buffer. Salt-extracted H3:H4 complexes in 2.0 M NaCl, pH 7 phosphate buffer were dialyzed exhaustively against 10.0 mM sodium phosphate and 0.25 mM EDTA, pH 7.0, at 4 °C. The CD spectrum of the salt-extracted complex in this buffer is shown in Figure 6a. The spectrum of the acid-renatured complex in the same buffer is identical within experimental error:  $[\theta]_{208} = -12\,800$  deg cm<sup>2</sup>/dmol and  $[\theta]_{222} = -9400$  deg cm<sup>2</sup>/dmol. Note that removal of NaCl from the solution of H3:H4 at pH 7 resulted in substantial changes in ellipticity at both extrema, compared to the high-salt spectra of Figure 3. Salt-extracted and acid-renatured H3:H4 complexes in 2.0 M NaCl, pH 7 phosphate buffer were also dialyzed exhaustively against 50 mM NaOAc and 0.25 mM EDTA, pH 5.0, at 4 °C. The peptide CD spectra of the salt-extracted complex in the pH 5 buffer is shown in Figure 6b. The spectrum of the renatured complex was also identical within experimental error:  $[\theta]_{203} = -13\,900$  deg cm<sup>2</sup>/dmol and  $[\theta]_{222} = -8000$  deg cm<sup>2</sup>/dmol. The salt-extracted H3:H4 complexes (in pH 7 phosphate buffer and pH 5.0 acetate buffer) were renatured to 2.0 M NaCl, 10.0 mM sodium phosphate, and 0.25 mM EDTA, pH 7, by exhaustive dialysis at 4 °C. The resulting spectra (Figure 6c,e) are identical with the original spectra in 2.0 M NaCl, pH 7 phosphate buffer obtained before cycling through low

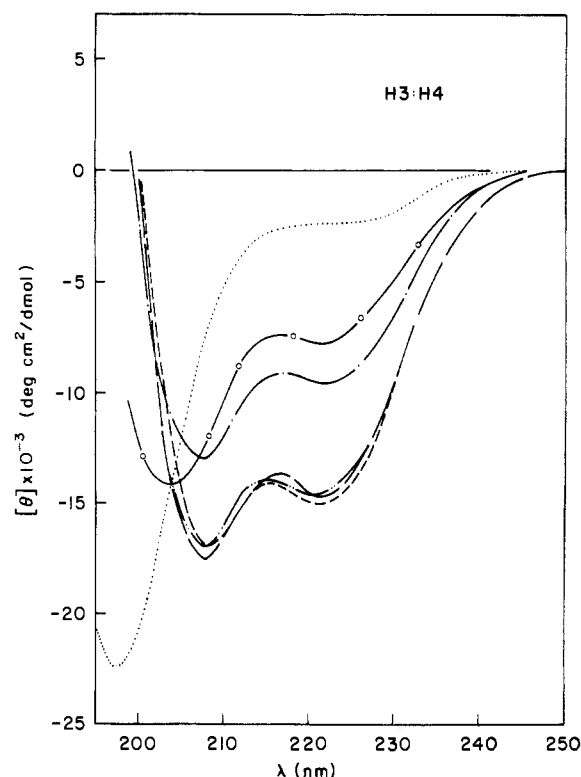


FIGURE 6: Circular dichroism spectra of the peptide region of salt-extracted H3:H4 complexes exposed to various solvent conditions. Spectra were recorded in 0.2- or 0.5-mm cells at 4 °C after dialysis into the final buffer: (a) complex at 1 mg/mL in 10.0 mM sodium phosphate and 0.25 mM EDTA, pH 7.0 (---); (b) 1 mg/mL in 50.0 mM NaOAc and 0.25 mM EDTA, pH 5.0 (—○—); (c) 0.22 mg/mL in 1.0 mM HCl; (···); (d, e) ~1 mg/mL in 2.0 M NaCl, 10.0 mM sodium phosphate, and 0.25 mM EDTA, pH 7.0, after exposure for at least 2 days to (d) 10.0 mM sodium phosphate and 0.25 mM EDTA, pH 7.0 (---), or (e) 50.0 mM NaOAc and 0.25 mM EDTA, pH 5.0 (---); (f) 0.43 mg/mL in 2.0 M NaCl, 10.0 mM sodium phosphate, and 0.25 mM EDTA, pH 7.0, after exposure for at least 2 days to 1 mM HCl followed by exposure to 10 mM sodium phosphate, pH 7.0, for 1 day (---).

salt (Figure 3). Salt-extracted H3:H4 was also examined after extensive dialysis into 1.0 mM HCl (Figure 6c). The CD spectrum ( $[\theta]_{198} = -22\,500$  deg cm<sup>2</sup>/dmol and  $[\theta]_{222} = -2400$  deg cm<sup>2</sup>/dmol) is essentially random coil. Upon dialysis, from 1.0 mM HCl to 10.0 mM sodium phosphate, 0.25 mM EDTA, and 0.1 mM DTT, pH 7, and then to 2.0 M NaCl, pH 7 phosphate buffer and 0.1 mM DTT, a spectrum (Figure 6f) identical with the original high-salt spectrum was obtained.

Complexes of salt-extracted and acid-renatured H2A:H2B were also examined under the same regimen of buffer conditions and tested for conformational reversibility upon return to 2.0 M NaCl, pH 7 phosphate buffer. Salt-extracted and acid-renatured H2A:H2B complexes in 2.0 M NaCl, pH 7 phosphate buffer were dialyzed exhaustively against 10.0 mM sodium phosphate and 0.25 mM EDTA, pH 7.0, at 4 °C. The peptide CD spectra of these two complexes in the NaCl-free buffer were identical within experimental error:  $[\theta]_{208} = -17\,000$  deg cm<sup>2</sup>/dmol and  $[\theta]_{222} = -14\,400$  deg cm<sup>2</sup>/dmol (Figure 7a). Salt-extracted and renatured H2A:H2B complexes in 2.0 M NaCl, pH 7 phosphate buffer were also dialyzed against 50 mM NaOAc and 0.25 mM EDTA, pH 5.0, at 4 °C. The CD spectra of these complexes in pH 5 buffer were also identical:  $[\theta]_{202} = -15\,500$  deg cm<sup>2</sup>/dmol and  $[\theta]_{222} = -4200$  deg cm<sup>2</sup>/dmol (Figure 7b). These complexes have lost considerable amounts of their secondary structure. The salt-extracted H2A:H2B complexes (in pH 7 phosphate buffer and pH 5.0 acetate buffer) were returned to 2.0 M NaCl, 10.0



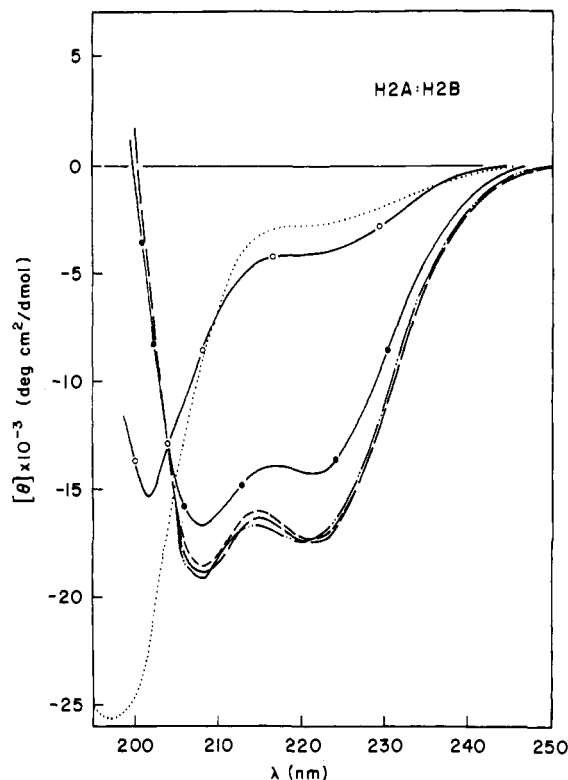


FIGURE 7: Circular dichroism spectra of the peptide region of salt-extracted H2A:H2B complexes exposed to various solvent conditions; the figure symbols represent the same solvent conditions as those of the legend to Figure 6, except that the protein concentration in 1.0 mM HCl is 0.365 mg/mL.

mM sodium phosphate, and 0.25 mM EDTA, pH 7.0, by exhaustive dialysis at 4 °C. The CD properties of these samples (Figure 7d,e) approached the spectral characteristics of the original spectra obtained before the samples were cycled through low salt (Figure 4). Salt-extracted H2A:H2B was also examined after extensive dialysis into 1.0 mM HCl (Figure 7c). Like H3:H4, the spectrum ( $[\theta]_{198} = -25\,700$  deg cm<sup>2</sup>/dmol and  $[\theta]_{222} = -2800$  deg cm<sup>2</sup>/dmol) is also essentially random. Upon dialysis from 1 mM HCl to 2.0 M NaCl, pH 7 phosphate buffer, through the intermediate low salt, pH 7 phosphate buffer, the original high-salt spectrum was obtained (Figure 7f).

The same cycling regimen was applied to the core histone complex, and essentially complete reversibility of spectral characteristics could be demonstrated. The CD spectrum of the core histone complex in 2.0 M NaCl, pH 7 phosphate buffer is shown in Figure 8a. In 1.0 mM HCl, the CD spectrum of the core complex (Figure 8b) indicates essentially complete random structure. When the complex is dialyzed back from 1.0 mM HCl to 2.0 M NaCl, pH 7 phosphate buffer and 0.1 mM DTT, through the intermediate low salt, pH 7 phosphate buffer, the original high-salt spectrum was obtained (Figure 8c).

Thus all the histone complexes examined appear by the criterion of similar CD spectra to be capable of total renaturation to a relatively structured state, even after being exposed to partial (low salt, pH 5 or 7), extensive (1.0 mM HCl), or severely denaturing (acid extraction) conditions.

**Estimation of Secondary Structure by Analysis of Circular Dichroism Spectra.** The peptide CD spectra of salt-extracted and renatured histone complexes presented above were fit to linear combinations of  $\alpha$ -helical,  $\beta$ -sheet, and random coil secondary structures by using the two sets of basis spectra which are most commonly used in the literature. Spectra for

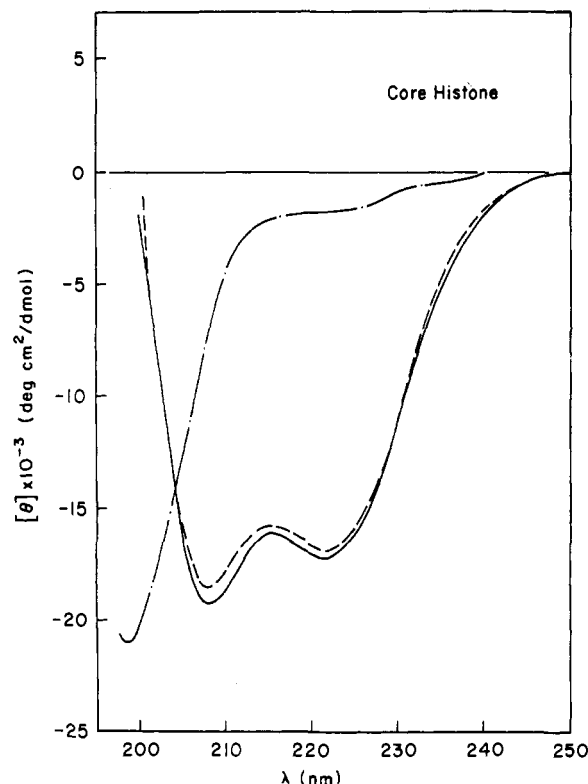


FIGURE 8: Circular dichroism spectra of the peptide region of native denatured and renatured salt-extracted core histone complex. Spectra were recorded in 0.2- or 0.5-mm cells at 4 °C. (a) Freshly purified core in 2.0 M NaCl, 10.0 mM sodium phosphate, and 0.25 mM EDTA, pH 7.0, 0.60 mg/mL (---); (b) salt-extracted core in 1.0 mM HCl, 0.25 mg/mL (- - -); (c) salt-extracted core in 2.0 M NaCl, 10.0 mM sodium phosphate, and 0.25 mM EDTA, pH 7.0, after 2 days of dialysis in 1.0 mM HCl and 1 day of dialysis in 10.0 mM sodium phosphate and 0.25 mM EDTA, pH 7.0, 0.46 mg/mL (—).

poly(L-lysine) in the  $\alpha$ -helical and  $\beta$ -sheet conformations (Greenfield & Fasman, 1969) were combined with the averaged spectra of the individual core histones in 1.0 mM HCl to represent the random coil state. Curve fitting was carried out from 203 to 250 nm. It has been our observation that the poly(L-lysine)-histone reference spectra generally lead to lower estimates of  $\alpha$  helix and higher estimates of  $\beta$  sheet than reference spectra derived from proteins (Chen et al., 1972). In general, the poly(L-lysine)-histone standards provide better fits than the protein standards at wavelengths below 215 nm. The converse is usually true for wavelengths above 215 nm. It should be emphasized that changes in predicted  $\alpha$  or  $\beta$  content are generally more reliable than the absolute values of these numbers.

In these studies, DTT has not been uniformly used, although in the latter experiments it was routinely included. However, similar results were obtained in the CD studies, with or without its use. It is advisable to include it to prevent disulfide formation. Similarly the absence of PMSF in the preparation of the chromatin did not lead to degradation of histones in the methods employed. However, for safety, this was routinely added at the latter stages of this research.

#### Discussion

It has been known for several years that individually purified core histones, isolated by denaturing methods, can either self-assemble into large polymeric structures or associate with each other to form small heterocomplexes (D'Anna & Isenberg, 1974a and references therein; Sperling & Bustin, 1975 and references therein). The biological significance of large histone polymers is not understood at present. Although



models have been proposed to accommodate these polymers into the formation of chromatin (Hyde & Walker, 1975; Sperling & Bustin, 1975), our present understanding of chromatin structure does not lead to an obvious correlation.

Methods for the preparation of large amounts of homogeneous renatured histone complexes from the individual components, free of histone polymers, were described by Nicola et al. (1978). The present study assesses the "nativeness" of renatured complexes by comparing their chromatographic and CD spectral properties with those of the complexes prepared by mild salt-extraction methods. A summary of various methods is discussed by Simon & Felsenfeld (1979). The H3:H4 tetramer, the H2A:H2B dimer, and the core histones, whether prepared by renaturation of acid-extracted histones or salt extraction on agarose columns, exhibit identical behavior by gel exclusion chromatography (Figure 2).

Within experimental error, the CD spectra of H3:H4 and H2A:H2B complexes are independent of the methods of preparation employed in this study (see Materials and Methods). In addition, perturbation of the environment to low salt at pH 5 or 7 followed by reequilibration to 2.0 M salt, pH 7 phosphate buffer does not lead to an irreversible change in apparent secondary structure. Moss et al. (1976a,b) have reported physical studies on the H3:H4 tetramer and H2A:H2B dimer isolated by the method of Van der Westhuyzen & Von Holt (1971). They found that H2A:H2B complexes in 50 mM NaOAc and 50 mM NaHSO<sub>3</sub>, pH 5.7, at room temperature, exists in a conformational state containing 38%  $\alpha$ -helical and essentially no  $\beta$ -sheet structures by CD [using basis spectra of Chen et al. (1972)] and infrared spectroscopy; this complex could undergo partial reversible denaturation. The same conformation was found for this complex in 25 mM potassium phosphate, pH 7.0, at room temperature. The data reported herein differ in that a pH dependence of conformation is reported. The H2A:H2B complexes at pH 5.0, 4 °C (50 mM NaOAc and 0.25 mM EDTA), existed as a random coil (Table II), while at pH 7.0, 4 °C (10 mM sodium phosphate and 0.25 mM EDTA), the conformation was identical with that reported by Moss et al. (1976a) at room temperature. Perhaps the differences can be attributed to the difference in temperatures, the difference in pH, and the higher ionic strength present in the buffer used. Moss et al. (1976b) reported that the H3:H4 tetramer at pH 5.0 (50 mM NaOAc and 50 mM NaHSO<sub>3</sub>) at room temperature contained 29%  $\alpha$ -helical structure; in the presence of 1 M NaCl in the above buffer, a helical content of 35% was found. The H3:H4 complex studied herein had a lower helical content (13%) at pH 5.0, 4 °C (50 mM NaOAc and 0.25 mM EDTA) (Table II), while in the presence of 2.0 M NaCl (10 mM sodium phosphate, 0.25 mM EDTA, and 0.1 mM DTT) at pH 7.0, 4 °C, the helical content was 35%, similar to that reported above for a 1 M NaCl solution. Moss et al. (1976b) reported H3:H4 aggregation at salt concentrations greater than 300 mM NaCl whereas in the present study 2 M NaCl was routinely used. Thus differences are again observed which may be attributed to differences in temperature, ionic strength, and protein concentration ( $\sim 7$  mg/mL Moss et al. and 1–1.7 mg/mL herein), which may cause reversible dissociation (Roark et al., 1974) and affect the secondary structure. The basic CD spectra used to analyze the curves also produce differences in calculated conformation (see Table II).

Lewis (1976) reported CD spectra of H3:H4 complexes under varying ionic strength conditions which are in basic agreement with those reported herein. In 50 mM phosphate, pH 7, he reports a 28%  $\alpha$ -helical content, using the  $\alpha$  and  $\beta$

values of Chen et al. (1972) and the random values of H3:H4 in 1 mM HCl. This is in agreement with the values reported in Table II. However, he found irreversibility in cycling through acid pH, which he attributed to oxidation of the SH group of H3. When DTT is used as demonstrated herein, complete reversibility was observed, but only when dialysis proceeded through a low-salt step at neutral pH. Feldman et al. (1980) also have reported loss of helical content upon dilution of the H3:H4 complex from 2 M KCl (0.10 M potassium phosphate, pH 6.7) to 0.14 M NaCl (7.0 mM potassium phosphate, pH 7.4), in agreement with the studies discussed above.

The conformation of the core complex in 2 M NaCl, pH 7.0–7.5 (Thomas et al., 1977; Bidney & Reeck, 1977), appears to be similar to that found for the complex in chromatin at low ionic strength. It was estimated previously that the inner histone core (H2A:H2B:H3:H4)<sub>2</sub> had a helical content of approximately 50%  $\alpha$  with no  $\beta$  structure (Thomas et al., 1977). The conformation of the core histone complex studied herein, in 2.0 M NaCl, 10 mM sodium phosphate, 0.25 mM EDTA, and 0.1 mM DTT, pH 7.0, 4 °C, was found to agree with previously published values [wherein 47%  $\alpha$  and 7%  $\beta$  structure were found (Table II)]. The conformation of the core histone complex has been shown to be dependent on the ionic strength of the environment (Olins et al., 1977; Bidney & Reeck, 1977). Decreasing the ionic strength causes the secondary structure to slowly open to a much less ordered structure. The present study demonstrates that in 1 mM HCl the core histones have been completely denatured, with 0%  $\alpha$  (Table II), and they may be renatured to the original conformation in 2 M NaCl by the dialysis procedure outlined.

Bidney & Reeck (1977) have reported that the core histone complex isolated by a two-phase procedure employing high ionic strength and nonionic polymers displays CD properties identical with the renatured core histone complex previously acid extracted. Our results with various preparations of core also agree with this. However, it has been found that at high concentrations, core histone complexes display additional negative ellipticity which may indicate a conformational change due to a higher degree of association of the octamer (Godfrey et al., 1980; Stein & Page, 1980; Ruiz-Carrillo and Jorcano, 1979). A similar CD change with concentration has been reported by Philip et al. (1980); this will be discussed in a future publication (H. Okabayashi, N. V. Beaudette, and G. D. Fasman, unpublished results).

When Nicola et al. (1978) assembled their renatured complexes, they obtained considerable amounts of aggregated material, which was subsequently removed by gel exclusion chromatography. However, it is shown herein that H3:H4, H2A:H2B, and core complex can each be subjected to extensive ( $\sim 24$  h) dialysis in  $10^{-3}$  N HCl, under which conditions they appear to lose all  $\alpha$ -helical and  $\beta$ -sheet structure, and then renatured back to their original high-salt structures by dialysis against 2.0 M NaCl, phosphate buffer. For efficient renaturation, it is important that renaturation involve an intermediate dialysis into 10.0 mM sodium phosphate, 0.25 mM EDTA, and 0.1 mM DTT, pH 7, and that low protein concentrations be used ( $\leq 0.5$  mg/mL). Nicola et al. (1978) employed high protein concentrations, 10–15 mg/mL, in their procedure; it is likely that less aggregation would have occurred had a lower concentration been used.

The conformation of the core complex in 2 M NaCl (Thomas et al., 1977; Bidney & Reeck, 1977) appears to be similar to that found for the complex in chromatin in low ionic strength. Thus the conformation in 2 M NaCl may be de-

scribed as the native structure. This would agree with the studies of Weintraub et al. (1975) which indicated that the trypsin digestion of core histones in high salt was similar to that when associated with DNA.

The studies reported in this paper amply illustrate the conformational mobility of the core histone complex and the products of dissociation, the H2A:H2B dimer and H3:H4 tetramer. The complete reversibility from the random coil structure to the highly ordered structure found in chromatin (2 M NaCl conformation) provides the flexibility which may be necessary to convert inactive to active chromatin, as part of the mechanism involved in the transcriptional regulation of gene expression in eukaryotic cells [for review, see Mathis et al. (1980)].

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