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An Octamer of Core Histones in Solution: Central Role of the H3·H4 Tetramer in the Self-Assembly†

Adolfo Ruiz-Carrillo* and José Luis Jorcano

ABSTRACT: The association of histones H2A, H2B, H3, and H4 in solution has been studied. In 2 M NaCl and at neutral pH they can assemble in a complex in which each histone is present in equimolar amounts. The complex has a weight average molecular weight of 98 000 (±3700) and a sedimentation coefficient ($s^0_{20,w}$) of 4.8. The value of the weight average molecular weight and the histone stoichiometry indicate that the complex is an octamer. The pairs of histones H2A,H2B and H3,H4 studied separately under identical conditions only associate as equimolar complexes consistent with dimeric and tetrameric structures, respectively. The stability of the core histone octamer is a function of the ionic strength, pH, and concentration of protein. The octamer dissociates by losing dimers of H2A,H2B until the main complexes existing in solution are the H3·H4 tetramer and the H2A·H2B dimer. This process is reversible upon reestablishing the original conditions.

The nucleosome core particle has been shown to consist of approximately 140 base pairs of DNA complexed with the core histones (H2A, H2B, H3, and H4) (Sollner-Webb & Felsenfeld, 1975; Axel, 1975). In recent years evidence has accumulated in support of the view that the four core histones occur in the vast majority of the core particles (Kornberg & Thomas, 1974; Boseley et al., 1976; Camerini-Otero et al., 1976; Simpson & Bustin, 1976; Bustin et al., 1977; Ruiz-Carrillo & Jorcano, 1977; Oudet et al., 1977; Jorcano & Ruiz-Carrillo, 1979). In addition, hydrodynamic (Shaw et al., 1976) and cross-linking (Thomas & Kornberg, 1975a,b) studies have indicated that an octamer containing two of each core histone makes up the protein moiety of the core particle. Whether the core histone octamer can exist in the absence of DNA is at present a matter of controversy.

In solutions of low ionic strength, histones interact in pairs H2A,H2B (dimer) and H3,H4 (tetramer) (Kelley, 1973; Roark et al., 1974; Kornberg & Thomas, 1974), although a relatively strong cross-interacting dimer H4·H2B has also been characterized (D'Anna & Isenberg, 1974). The way in which the core histones associate in solution is, however, not well established. Weintraub et al. (1975) reported that in solutions of high ionic strength (i.e., 2 M NaCl) and neutral pH, they associate in "heterotypic" tetramers (H2A·H2B·H3·H4), a view later supported by laser light scattering studies (Campbell & Cotter, 1976). On the other hand, cross-linking studies have suggested that an octamer may exist under similar conditions (Thomas & Kornberg, 1975a,b). During the course of this work, Thomas & Butler (1977) have also reported hydrodynamic studies which are consistent with the existence of a core histone octamer in solution. A clarification of this controversy will contribute not only to our understanding of the structure of the nucleosome but also to the way in which chromatin is assembled during DNA replication. We present evidence demonstrating the occurrence of a core histone octamer in solutions of high ionic strength and describe some of its properties.

Experimental Procedures

Preparation of Cells and Nuclei. Mature hen erythrocytes were obtained as described previously (Ruiz-Carrillo et al., 1975). Cells were lysed in buffer A[1] (Hewish & Burgoyne,

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1973) containing 1% (v/v) Triton X-100 or Nonidet P-40 and 0.1 mM PMSF. Nuclei were pelleted by centrifugation and washed three to four times with the same buffer and finally once with 0.35 M NaCl, 0.1 mM PMSF, 10 mM Tris-HCl (pH 7.3). Polynucleosomes with an average molecular weight of 8×10^6 were prepared by micrococcal DNase digestion of nuclei (A. Ruiz-Carrillo, unpublished experiments).

Extraction of Histones. Unless so specified, all operations were carried out at 4 °C. Core histones (H2A, H2B, H3, and H4) were prepared by acid or salt extraction. Acid-extracted core histones were prepared after removal of H1 + H5 (Johns, 1964) by extraction in 0.25 N HCl and precipitation with acetone. Salt-extracted core histones were prepared from washed nuclei after removal of H1 + H5 in 0.75 or 0.8 M NaCl by extraction of the residual nucleohistone in 2 M NaCl, 0.5 mM EDTA, 0.1 mM PMSF, and 5 mM Tris-HCl (pH 7.3) for 10 h. DNA and residual debris were pelleted in a Spinco Ti50 rotor at 45 000 rpm for 18 h. The supernatant containing histones was concentrated by ultrafiltration in an Amicon cell (UM-2 filter) and dialyzed against 2 M NaCl in buffer B. Histone pairs H3,H4 and H2A,H2B were prepared from core histones by Sephadex G-100 column chromatography (180 × 5 cm diameter) at 4 °C, eluted with 0.2 M NaCl in buffer B, and concentrated by ultrafiltration.

Polyacrylamide Gel Electrophoresis. High-resolution polyacrylamide slab gel electrophoresis was carried out essentially according to the methods of Davis (1964) and Laemmli (1970) as modified by A. Ruiz-Carrillo (unpublished observations). The separating gel consisted of a 15–19% linear gradient of polyacrylamide with an acrylamide to bis(acrylamide) ratio of 30:0.8. $NaDodSO_4$ was omitted from both the stacking and the separating gels. The slabs were stained with Coomassie brilliant blue (Sigma). An Ortec 4310 densitometer (Ortec Inc.) coupled to an automatic integrator was used for quantitative measurements of the bands in the gels. The relative amount of each histone was estimated from its area using as a reference sonicated nuclei or soluble polynucleosomes run in the same slab (Figure 1). The stoichiometry of the nuclear core histones was assumed to be equimolar (Joffe et al., 1977). Control experiments in which nuclear proteins were radioactively labeled showed that by this procedure all the radioactivity entered the gel (not shown). Since the ratios of the areas of H3/H4 and H2A/H2B were found to be constant in the stoichiometric complexes analyzed and equal to those found in nuclear histones, they were arbitrarily assigned a value of 1 (see Figure 2B). The ratio of (H3 + H4)/(H2A + H2B) was used to determine the stoichiometry of the core histone complexes. Again, a value of 1 indicates that each histone pair was present in the complex in the same relative amount as in nuclei or soluble polynucleosomes (Figure 1).

Reductive Alkylation. Protein standards, bovine serum albumin, ovalbumin, horse myoglobin, ferritin, and catalase were radioactively labeled with C^3H_2O (NEN 5 mCi/1.5 mg) by reductive methylation according to Rice & Means (1971) as modified by Ruiz-Carrillo et al. (1975). The elution in gel filtration columns and the S value of the labeled markers were unaffected by this treatment.

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; EDTA, disodium salt of ethylenediaminetetraacetic acid; buffer A, 0.34 M sucrose, 0.5 mM spermidine, 0.15 mM spermine, 60 mM KCl, 10 mM Tris-HCl, pH 7.2; buffer B, 0.01% (v/v) 2-mercaptoethanol, 0.2 mM PMSF, 5 mM Tris-HCl, pH 7.2; $NaDodSO_4$, sodium dodecyl sulfate; core histones, H2A, H2B, H3, and H4.

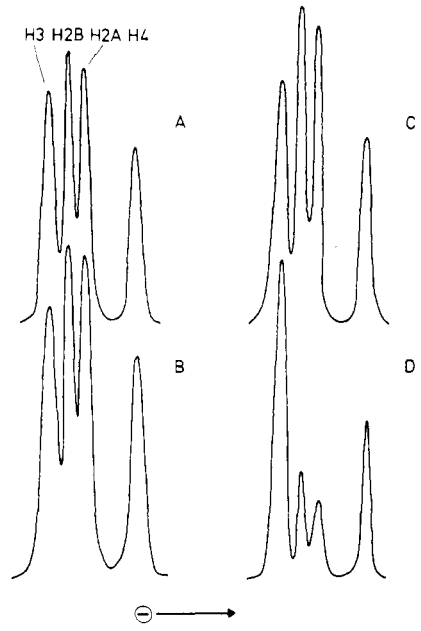

FIGURE 1: Stoichiometry of core histones in the nucleus and in the core histone complex. Densitometer tracings of core histones separated by $NaDodSO_4$ polyacrylamide gel electrophoresis. (A) Nuclei or soluble polynucleosomes; (B) core histone complex prepared by Bio-Gel A-0.5 m (see Figure 2A), (C) typical profile of extracted core histones; (D) nuclear residue after the extraction of core histones (C). The ratio (H3 + H4)/(H2A + H2B) was 0.97 (A), 1.05 (B), and 0.88 (C). For further details, see Experimental Procedures.

Sucrose Gradient Sedimentation. Core histone complexes were analyzed in 5–20% (w/v) linear sucrose gradients at the same ionic strength and pH as that of the loaded sample. Gradients were centrifuged for 38 to 41 h at 56 000 rpm in a Spinco SW 60Ti rotor at 3 °C. Radioactively labeled protein standards were run as internal markers and also in parallel tubes. The sedimentation profiles were obtained after adding to each fraction (100 μL) trichloroacetic acid to a final concentration of 18% (w/v) and measuring their turbidity at 320 nm after 30 min. Aliquots of each fraction were analyzed by $NaDodSO_4$ polyacrylamide gel electrophoresis.

Gel Filtration Chromatography. Histone complexes were analyzed by gel permeation chromatography (column type K 15/90, Pharmacia) at 4 °C or 20 °C in Bio-Gel P200 (100–200 mesh) and Bio-Gel A-0.5 m (200–400 mesh) (Bio-Rad Laboratories). Void volume (V_0) was determined either with ^{3}H-labeled *Escherichia coli* DNA (a kind gift of Dr. R. Synenki) included as internal marker in some of the runs in 2 M NaCl (Bio-Gel A-0.5 m) or with ^{3}H-labeled ferritin (Bio-Gel P200). The amount of histone loaded onto the column varied between 0.3 and 9 mg in 1 mL. Radioactively labeled protein standards were run as internal markers. The elution profile was determined by turbidimetry. Aliquots of each fraction (1 mL) were analyzed by $NaDodSO_4$ polyacrylamide gel electrophoresis as described above. Apparent molecular weights (M_{app}) were calculated by reading off the plot of the partition coefficient, K_{av}, vs. log M of the protein standards (Laurent & Killander, 1964). In the case of the Bio-Gel A-0.5 m it was found that this relationship was essentially linear, at least between 116 K (dimer of catalase) and 17.8 K (myoglobin). The Stokes radius R_0 was determined by reading off the plot of $(-\log K_{av})^{1/2}$ vs. R_0 for the standard proteins (Siegel & Monty, 1966). The frictional ratio was calculated using $f/f_0 = R_0/(3\bar{\nu}M/4\pi N)^{1/3}$, where M = molecular weight, $\bar{\nu}$ = partial specific volume, and N = Avogadro's number.

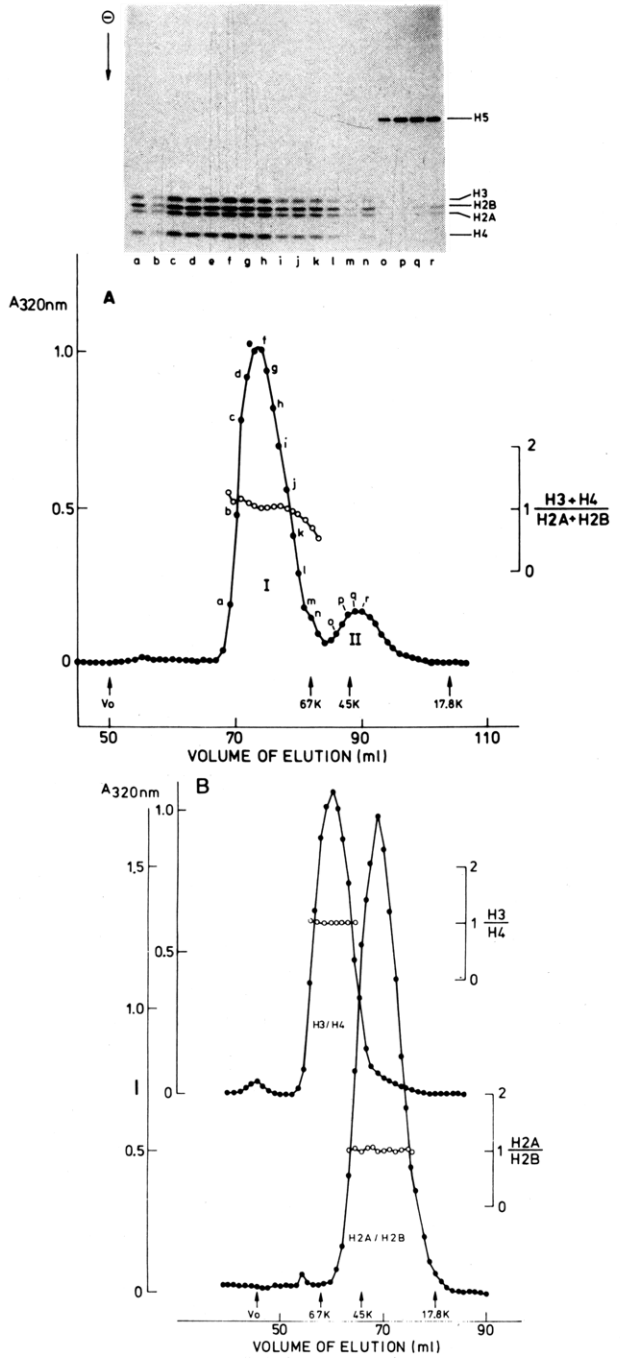

FIGURE 2: Association of histones H2A, H2B, H3, and H4 in solution. (A) Salt-extracted core histones in 2 M NaCl (buffer B, pH 7.2) (8.8 mg in 1 mL) were chromatographed at 4 °C in Bio-Gel A-0.5 m eluted with the same buffer. Before loading, histones were mixed with tracer amounts of ^{3}H-labeled markers: *E. coli* DNA (V_0), bovine serum albumin (67 000), ovalbumin (45 000), horse myoglobin (17 800). The elution profile was determined by turbidimetry at 320 nm (●–●). The peak fraction of the elution of the markers (indicated by the arrows) was determined by scintillation counting. Fractions were analyzed in 15–19% NaDodSO$_4$ polyacrylamide gels as indicated. (O–O) Ratio of (H3 + H4)/(H2A + H2B). Acid-extracted core histones showed similar elution pattern when analyzed under the same conditions. (B) Histone pairs H2A,H2B (0.7 mg in 1 mL) or H3,H4 (0.5 mg in 1 mL) in 2 M NaCl (buffer B, pH 7.2) were chromatographed in Bio-Gel P-200 eluted with the same buffer. Tracer markers were as in A except that V_0 was determined with [^{3}H]ferritin. (O–O) Ratio of each histone in a pair. The rest of symbols are as in A. For further details, see Figure 1.

Determination of Molecular Weight and Other Physical Parameters. Core histones dialyzed for 48–72 h at 4 °C against 2 M NaCl in buffer B (pH 8.0) were used in all the experiments. The weight average molecular weight of the histone complex was determined by sedimentation equilibrium. One hundred microliters of sample and buffer was loaded in an AN-G rotor using standard cells fitted with 12-mm center pieces. Sedimentation was at 23 000 rpm and 12 °C (±0.1 °C) in a Beckman Model E ultracentrifuge equipped with a photoelectric multiplex scanner. Scans at 280 nm were recorded at 12 and 26 h from the start of the centrifugation. Samples were virtually at equilibrium after 12 h, but the data were taken from the 26-h scans.

Sedimentation velocity experiments were carried out at 12.5 °C at 40 000 rpm (rotor AN-G) using the UV scanner. Viscosity measurements were made at 12.5 °C (±0.01 °C) with an Ostwald type microcapillar viscosimeter coupled to an automatic measuring system (AVS/G, Schott Glass Co., Mainz, Federal Republic of Germany). The density of the protein solutions and buffers was measured in a precision digital densitometer (DMA O2C, Anton Paar) at 12.5 °C (±0.01 °C) (Kratky et al., 1973), calibrated with air and double distilled, Millipore filtered water. A value of 0.756 mL/g for the partial specific volume ($\bar{\nu}$) was obtained from the slope of the plot of the density of the solution vs. the concentration of protein (Figure 4B). Core histone concentrations were determined from the amount of amino acids recovered after hydrolysis in sealed evacuated tubes with 5.7 N HCl, 0.05% phenol for 22 h at 110 °C. The hydrolysates were analyzed in a Durrum 500 analyzer coupled to a Teleprint UCCG computer. Norleucine was used as internal standard, and no corrections for hydrolytic losses were made. The value for the absorption coefficient ($A_{275}^{1\%} = 4.3$) obtained by this method was in excellent agreement with those obtained by nitrogen determination (Jaenicke, 1974; $A_{275}^{1\%} = 4.3$) and microbiuret (Goa, 1953; $A_{275}^{1\%} = 4.6$) using ovalbumin (Pentax, Miles) as standard. Duplicate and triplicate determinations at several dilutions were made.

Results

Size and Stoichiometry of the Core Histone Complex. The complexes formed by the core histones, H2A, H2B, H3, and H4, were examined by gel filtration chromatography. Figure 2A shows a typical elution profile of salt-extracted core histones in 2 M NaCl (pH 7.2). About 88% of the protein eluted in peak I. The apparent molecular weight (M_{app}) of the complex was 120 000. Virtually no material eluted between the void volume and peak I, indicating the absence of larger aggregates. The four core histones occurred in the complex (Figure 2A, inset). The histone composition in each fraction is expressed as the ratio of the area of H3 + H4 over that of H2A + H2B. A ratio of 1 indicates that the stoichiometry of histones in the soluble complex and in the nucleus is the same (see Experimental Procedures). This quantitative analysis confirmed that in peak I the four core histones were present in equimolar amounts (Figure 1A,B and Figure 2A). The ratio (H3 + H4)/(H2A + H2B) deviated from 1 at the front and trailing boundaries due to a concentration-dependent dissociation of the complex (see below). The presence of a system in equilibrium was also evidenced by the shape of the advancing (sharp) and trailing (spread) edges. Peak II (12%) is a mixture of contaminating H5 (8%) and dimers of H2A, H2B (4%), which varied from preparation to preparation. The amount of H2A,H2B recovered in peak II was partly due to its excess over H3,H4 in the analyzed sample because of its easier extractability both in salt and in acid (Figure 1C,D; see also Ruiz-Carrillo et al., 1974). An identical association behavior was also observed when acid-extracted and acetone-precipitated core histones were analyzed under the same

Table I: Physical Parameters of Histone Complexes

	H2A·H2B dimer	H3·H4 tetramer	octamer
M^a	27 800	53 100	108 600
$M_{app}{}^b$	32 000	58 000	115 000
$M_w{}^c$			98 000 ± 3700
$S_{app}{}^d$	2.3	3.2	4.4
$s^0{}_{20,w}$			4.8
R_0 (Å)^e	26	31.5	40
$f/f_0{}^e$	1.27	1.23	1.23

[a] Calculated for calf thymus histones (reviewed in Elgin & Weintraub, 1975). [b] Determined by filtration chromatography. [c] Determined by equilibrium sedimentation. [d] Obtained by centrifugation in sucrose gradients. [e] From gel filtration (Siegel & Monty, 1966).

conditions (not shown). The same results were obtained at 4 and 20 °C (A. Ruiz-Carrillo and J. L. Jorcano, unpublished observations).

The pairs of histones H3,H4 and H2A,H2B chromatographed separately in 2 M NaCl (pH 7.2) (Figure 2B) eluted as equimolar complexes with M_{app} of 58 000 and 32 000, respectively, consistent with tetrameric (H3,H4) and dimeric (H2A,H2B) structures. These are the same type of complexes as found at lower ionic strength and/or acid pH (see below, and van der Westhuyzen & von Holt, 1971; Kelley, 1973; Roark et al., 1974; Kornberg & Thomas, 1974; D'Anna & Isenberg, 1974). The association behavior of these histone pairs was not dependent on the protein concentration range studied (see Figure 3B,C).

The M_{app} of the core histone complex in 2 M NaCl is compatible with an octamer or with a very asymmetric tetramer which would behave anomalously in gel filtration. The latter is, for instance, the case for H5 that has an average M_{app} of 36 000 under the same conditions of analysis (Weintraub et al., 1975; A. Ruiz-Carrillo and J. L. Jorcano, unpublished results). This possibility was ruled out by examining the hydrodynamic behavior of the complex. Figure 3A shows that by sedimentation in sucrose gradients it moved faster than its contaminants as a single band in which each histone was present in equimolar amounts (see Figure 3A, inset). The S_{app} value found for the complex (4.4) was higher than those found for the tetramer of H3,H4 (3.2) and the dimer of H2A,H2B (2.3) (Figure 3B,C and Table I). If the complex observed by gel filtration had been a very asymmetric tetramer, it should have given a S_{app} value lower than that of the H3·H4 tetramer (see for instance this behavior in the contaminating H5). Judging from the stoichiometry of the histones and from both the M_{app} and S_{app}, the preliminary conclusion is that the complex formed by the core histone is an octamer.

Molecular Weight and Hydrodynamic Properties. The weight average molecular weight (M_w) of the core histone complex in 2 M NaCl (pH 8.0) was determined by equilibrium sedimentation (Figure 4A). Only histone samples which were shown to assemble in stoichiometric complexes and which contained no or only small amounts of H5 contamination were used. Sedimentation equilibrium measurements were carried out at four different protein concentrations between 4.8 and 0.47 mg/mL. Both salt- and acid-extracted core histones gave the same slope from the plot of ln (A_{280}) vs. the radius squared (r^2). The average M_w obtained in the protein concentration range studied was 98 000 (±3700). This value is very close to the one expected for an octamer (see Table I) if we assume that the amino acid sequence of chicken erythrocyte core histones is identical with that of the calf thymus core histones (reviewed in Elgin & Weintraub, 1975). Thus, we shall refer

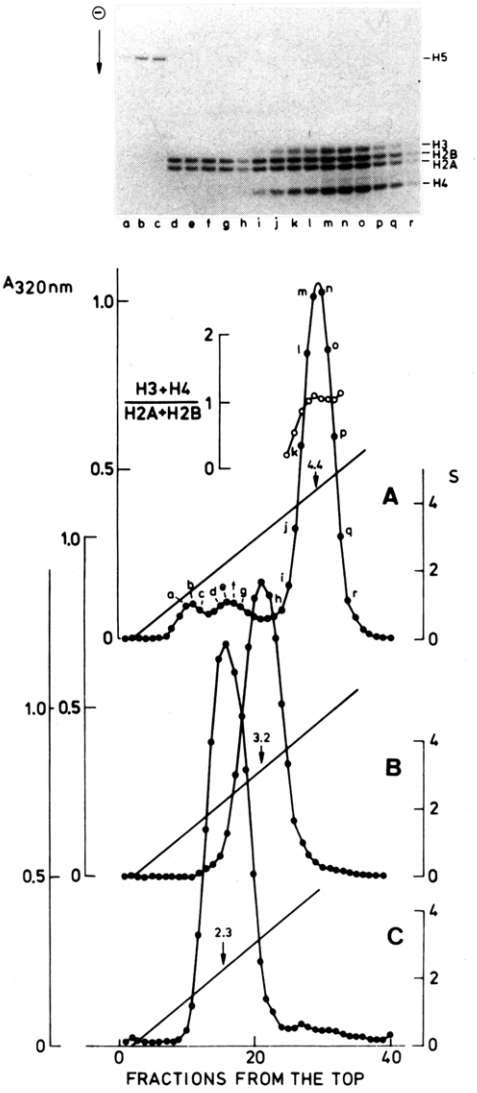

FIGURE 3: Sedimentation analysis of histone complexes. Core histones and histone pairs H2A,H2B and H3,H4 in 2 M NaCl (buffer B, 7.2) were analyzed by velocity sedimentation in 5–20% sucrose gradients in the same buffer. ^{3}H-labeled standard proteins, horse myoglobin (2.04 S), ovalbumin (3.55 S), and bovine serum albumin (4.5 S), were run as internal sedimentation markers. In parallel gradients, lysozyme (1.91 S) and human IgG (7.05 S) were also run. Centrifugation was in a Spinco SW 60Ti at 3 °C for 41 h at 56 000 rpm. Fractions, as indicated, were analyzed in 15–19% NaDodSO$_4$ polyacrylamide gels. The continuous line across the gradients is a plot of the $s^0{}_{20,w}$ value for the standard proteins as a function of the distance sedimented. The arrows indicate the S_{app} values obtained for the histone complexes as read off the standard plot. (A) Salt-extracted core histones (0.6 mg in 0.1 mL); (B) acid-extracted histones H3,H4 (0.25 mg in 0.1 mL); (C) acid-extracted histones H2A,H2B (0.25 mg in 0.1 mL). For further details, see legend to Figure 2.

hereafter to the complex made by H2A, H2B, H3, and H4 as an octamer. The core histone octamer showed a single symmetric sedimentation boundary in the range of concentrations analyzed (Figure 5A,B). The $s_{20,w}$ of the octamer (Figure 5B) extrapolates to $s^0{}_{20,w}$ of 4.8. No sign of dissociation of the complex was observed even at the lowest concentration used, in agreement with the estimates obtained by column chromatography (see below).

Stability of the Complex

(*a*) *Protein Concentration Dependency.* The stability of the octamer in 2 M NaCl and at neutral pH is a function of the concentration of protein. The M_{app} obtained for the complex by sieving chromatography decreased on lowering the amount

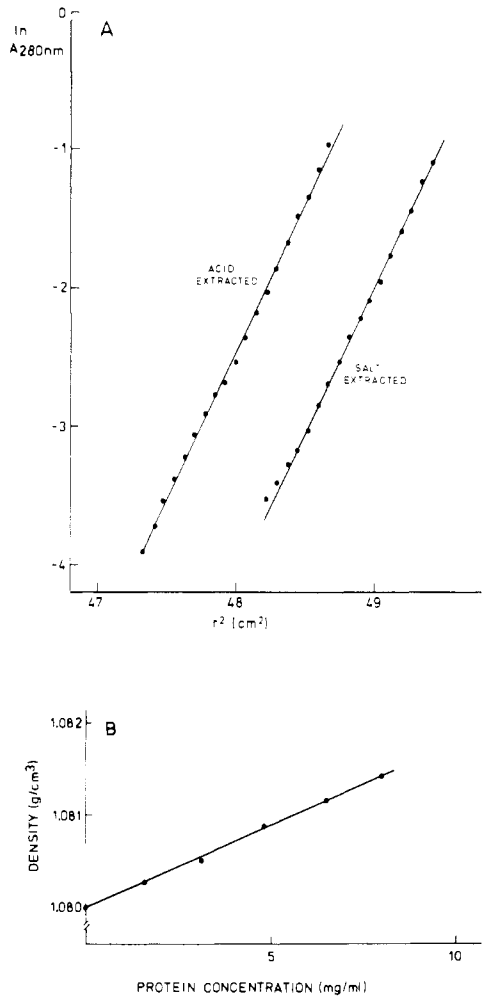

FIGURE 4: Equilibrium sedimentation and density measurements. (A) Plots of the ln (A_{280}) against the radius squared of the core histone complex. Samples of core histones showing equimolar stoichiometry were sedimented to equilibrium at 23 000 rpm at 12 °C (±0.1 °C) in 2 M NaCl (buffer B, pH 8.0). The lines through the experimental points were fitted by least squares. Acid- and salt-extracted core histones were at 4.8 and 1.0 mg/mL, respectively. (B) Densities of the core histone complex in 2 M NaCl (buffer B, pH 8.0) at 12 °C (±0.01 °C). The line through the experimental points was fitted by least squares. The value for the partial specific volume derived from this data was 0.756 mL/g.

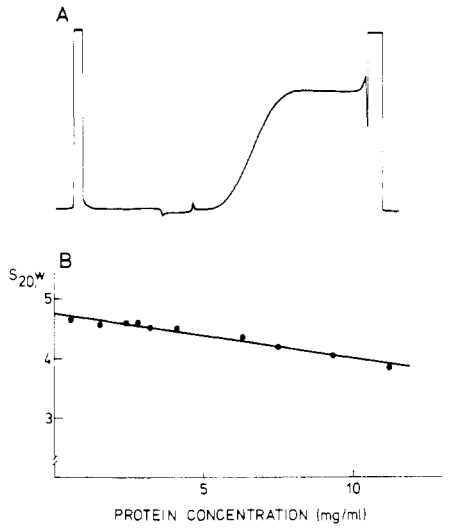

FIGURE 5: Sedimentation of the core histone octamer. Core histones in 2 M NaCl (buffer B, pH 8.0) were centrifuged at 40 000 rpm at 12.5 °C (±0.1 °C). (A) Scanner trace of the sedimentation boundary of the core histone complex (2.4 mg/mL) taken 128 min after the start of the run. (B) $s^0_{20,w}$ determination of the core histone complex.

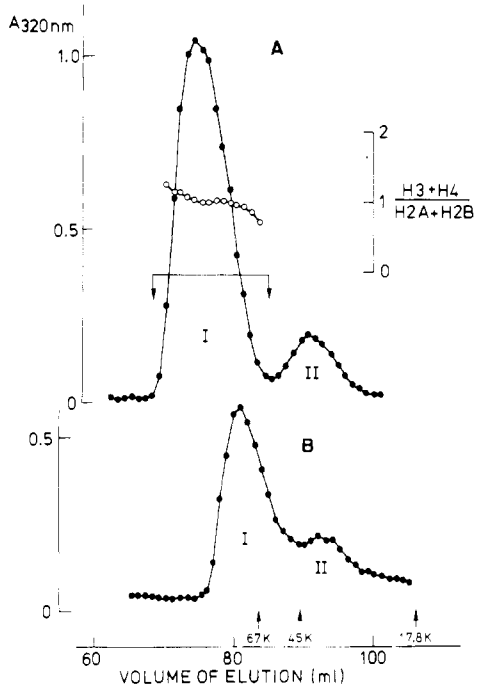

FIGURE 6: Protein concentration dependency of the core histone octamer. (A) Core histone octamer was prepared by chromatography in Bio-Gel A-0.5 m eluted with 2 M NaCl (buffer B, pH 7.2) as indicated by the arrows (histone loaded 8.8 mg in 1 mL). (B) An aliquot of the octamer (0.6 mg in 1 mL) was rechromatographed under the conditions described in A. The M_{app} of the complex in peak I was 72 000. Protein eluting in peak II was only H2A·H2B. See legend to Figure 2 for further details.

of histone loaded onto the column. Concomitantly, the amount of H2A·H2B appearing in peak II increased, while the overall ratio (H3 + H4)/(H2A + H2B) of the complex in peak I increased (see Figures 2A and 9). The change in these parameters indicates that the complex is dissociating. Figure 6 shows clearly the effect of lowering the concentration of protein. A complex with the characteristics of an octamer isolated by column chromatography as indicated (Figure 6A) was rechromatographed with a protein input 15-fold lower, and the result is shown in Figure 6B. The octamer had dissociated and the remaining complex eluted very asymmetrically with a M_{app} of 72 000, and a peak of dimers of H2A,H2B lost from the octameric structure (peak II) was visible. The stoichiometry of the histones in the complex in peak I revealed a clear excess of the pair H3,H4 over the H2A,H2B pair (result not shown). Since both filtration chromatography and sucrose gradient sedimentation involve a dilution of the sample, it is difficult to determine by these methods at what protein concentration the chemical equilibrium is clearly shifted toward the dissociation of the octamer. A rough estimation, however, could be obtained from the turbidimetric determination of the concentration of protein in chromatograms as the one shown in Figure 2A. This indicated that at around 0.2 mg/mL the ratio (H3 + H4)/(H2A + H2B) deviated from 1, suggesting that the complex was already unstable.

(*b*) *pH Dependency.* The octamer was found to be stable between pH 7 and 9 as judged from the M_{app} and S_{app} values and by the stoichiometry of the histones in the peak of the complex (not shown). In contrast it was very sensitive to acid pH. Dissociation was observed at pH 6 and was complete at

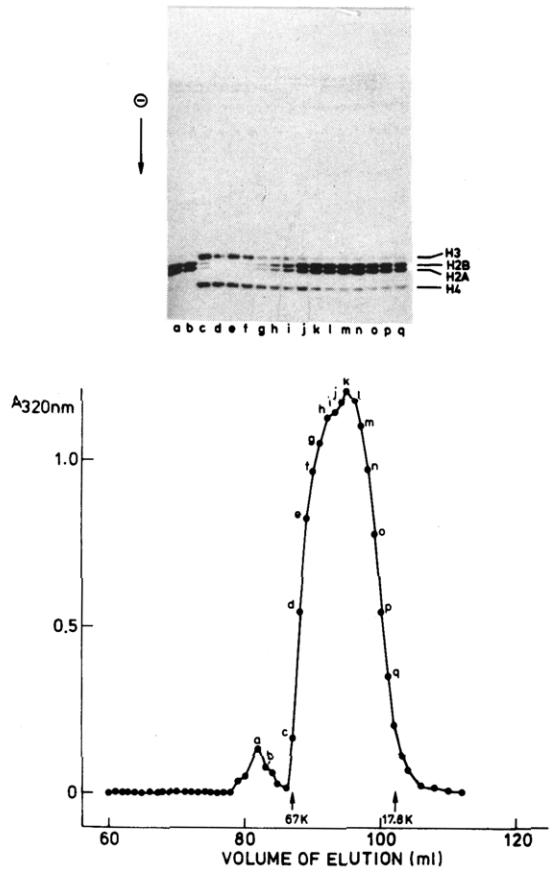

FIGURE 7: Dissociation of the core histone octamer at moderately acid pH. Core histones in 2 M NaCl (buffer B, pH 7.2) were dialyzed for 24 h against 2 M NaCl (5 mM sodium acetate, pH 5.0). Histones (3.5 mg) in 1 mL were loaded in a Bio-Gel A-0.5 m column and eluted with 2 M NaCl (pH 5.0). The inset shows the protein composition along the profile as analyzed by electrophoresis in 15–19% $NaDodSO_4$ polyacrylamide gels. For further details, see legend to Figure 2.

pH 5 (Figure 7). The octamer dissociated into H3·H4 tetramer and H2A·H2B dimer. The H3·H4 tetramer was also partly dissociated into dimers which were observed coeluting with the H2A·H2B dimer (Figure 7, n–q). Incidentally, a small fraction of H2A,H2B eluted ahead of the main peak as a discrete oligomeric complex (Figure 7, a,b). A similar oligomer of H2A,H2B was observed at neutral pH and lower ionic strengths (see Figure 8B, a,b). Since its proportion ranged from 0–3% of the total protein, it was not investigated further. The dissociation of the octamer caused by acid pH is reversible (result not shown).

(*c*) *Ionic Strength Dependency*. In an attempt to better characterize the self-assembly of the octamer, its pattern of dissociation was examined at intermediate salt molarities at neutral pH. Figure 8A shows the sedimentation properties of the octamer at 1.2 M NaCl. Under these conditions the S_{app} dropped from 4.4 to 4.2 and the ratio [(H3 + H4)/(H2A + H2B) ~ 1.6] of the complex was intermediate between that of an octamer and a hexamer (cf. Figures 3A and 8A). At the same time, the proportion of H2A,H2B sedimenting with the characteristics of the dimer increased. However, there was no H3,H4 either in the dimer or, possibly, in the tetramer region (Figure 8A, inset), suggesting that all the H3,H4 was still involved in the formation of higher order complexes. In 0.8 M NaCl and with a protein concentration similar to that of the experiment shown in Figure 9, the complex observed had a M_{app} of about 66 000 (Figure 8B). The stoichiometry of the complex in this case was compatible with that of an hexamer containing a H3·H4 tetramer and a H2A·H2B dimer [(H3 + H4)/(H2A + H2B) ~ 2] (Figure 8B, d–g). Although the amount of H2A·H2B lost to the dimer region increased (see Figures 8B and 9A), the majority of H3,H4 was again found in the complex, traces coeluting with dimer of H2A,H2B notwithstanding (Figure 8B, t–w). A further decrease in the salt molarity to 0.4 M NaCl completely dissociated the octamer (see Figure 9B). The results described above suggest that dissociation of the core protein octamer may occur via a hexameric structure as a result of the release of H2A·H2B dimers. Our results do not indicate, however, whether the hexamer is a stable complex or only a metastable intermediate. Next we investigated whether the dissociation by a decrease in the ionic strength of the core histone octamer is a reversible process. The results shown in Figure 9 show this to be the case. A sample of salt-extracted core histones, which due to the low input of protein (0.75 mg in 0.5 mL) eluted as a complex with M_{app} of 95 000 and an overall ratio (H3 + H4)/(H2A + H2B) of around 1.3, was dialyzed down to 0.4 M NaCl. At the lower ionic strength the complex was completely dissociated into H3·H4 tetramers and dimers and H2A·H2B dimers (Figure 9B). Upon dialysis back to 2 M NaCl the complex re-formed and behaved by gel filtration like the original sample (Figure 9C). In addition, the H3·H4 tetramer and the H2A·H2B dimer are the complexes found during renaturation of acid-extracted core histones in 0.2 or 0.4 M NaCl (pH 7.3) (results not shown). Thus, the reversibility of the core histone complex assembly and its pattern of dissociation under a variety of conditions also indicated that it always occurred via the same intermediate complexes.

Discussion

The four core histones exhibit a definite association behavior in solution. In low to moderately high ionic strength they interact as pairs. The pairs H3,H4 and H2A,H2B can associate as tetramers and dimers, respectively (van der Westhuyzen & von Holt, 1971; Roark et al., 1974; Thomas & Kornberg, 1974; Kelley, 1973; D'Anna & Isenberg, 1974; this work). We have shown that in these conditions the chemical equilibrium of the complex formation of each pair appears to be independent of the presence of the other. At high ionic strength and neutral pH, the isolated pairs still associate as H3·H4 tetramer and H2A·H2B dimer. This indicates that in the protein concentration range studied there are no stable interactions among tetramers of H3,H4 or among dimers of H2A,H2B. On the other hand, when the pairs H2A,H2B and H3,H4 are allowed to interact at neutral pH by an increase in the ionic strength, a cross-interacting complex can form (Weintraub et al., 1975; this work). This demonstrates that binding sites in the H3·H4 tetramer for the H2A·H2B dimer do occur, presumably, through affinity sites between H2B,H4 and H2A,H3 (D'Anna & Isenberg, 1974). The octamer is the largest stoichiometric complex of the core histones formed in 2 M NaCl (pH 7–9). The equilibrium of the assembly process is affected at least by the ionic environment, protein concentration, and pH, without significant effect of the temperature between 3 and 20 °C. An increase in the salt concentration may have two main stabilizing influences on the self-assembly of the octamer: (i) a favoring of the interaction through the apolar regions of the histones (Bradbury & Rattle, 1972; Böhm et al., 1977; Thomas et al., 1977) and (ii) a counteraction of the electrostatic repulsion of the positively charged N termini. The screening effect of the ionic strength may be analogous to that produced by the phosphate sugar backbone of the DNA. The gradual dissociation of the core histone octamer by a decrease in the ionic strength contrasts with its full dissociation at moderately acid pH. This suggests

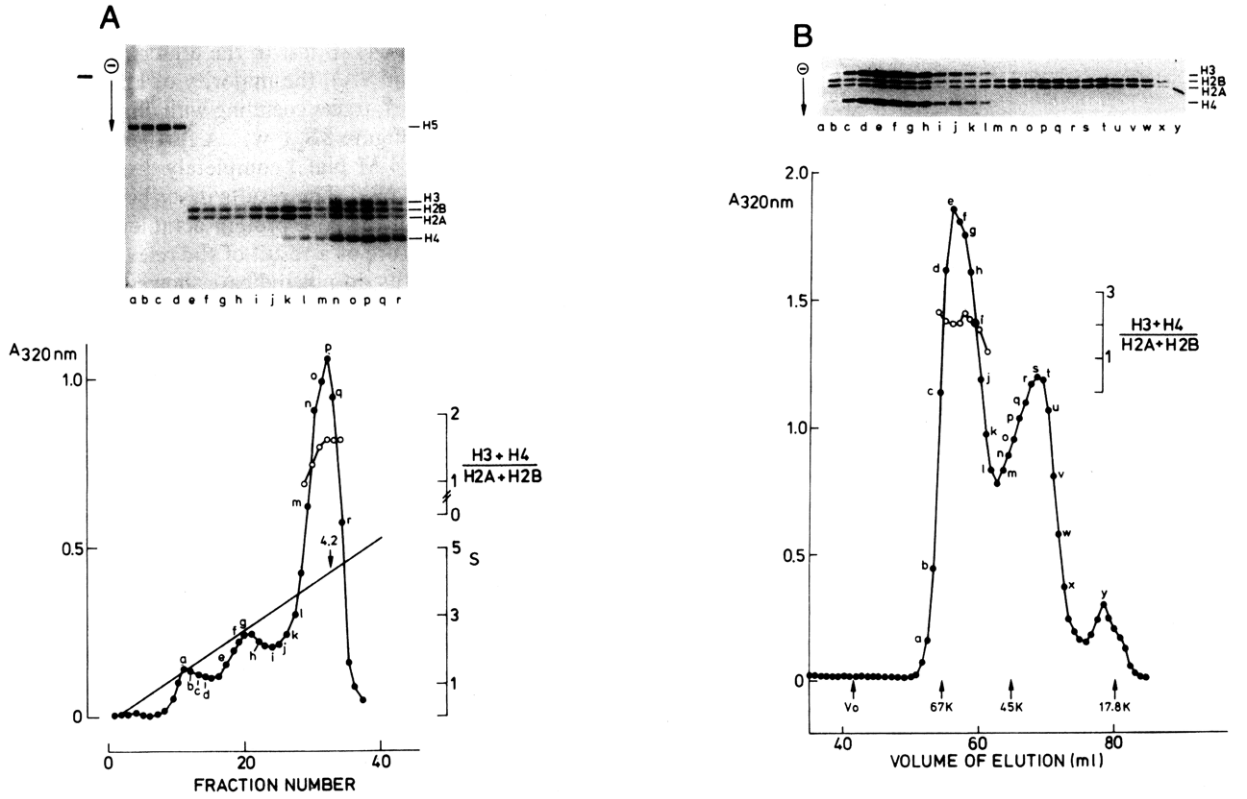

FIGURE 8: Dissociation of the core histone octamer as a function of the ionic strength. The stability of the core histone octamer at intermediate ionic strengths was examined by sedimentation in 5–20% sucrose gradients (A) and chromatography in Bio-Gel P-200 (B). Histones were first dialyzed extensively against 2 M NaCl (buffer B, pH 7.2) and then to the indicated ionic strength. (A) Sedimentation analysis of salt-extracted core histones in 1.2 M NaCl (buffer B, pH 7.2), protein input 0.6 mg in 0.1 mL. The inset shows the histone composition of the indicated fractions. Standard proteins were run as internal markers and in parallel tubes. For further details see legend to Figure 3. (B) Elution profile of core histones in 0.8 M NaCl (buffer B, pH 7.2), protein input 1.3 mg in 0.5 mL. The M_{app} of the complex under peak I was 64 000. The inset shows the protein composition of selected fractions. Peak III contained unpaired H2A (y), presumably in excess in the original sample. A small amount of oligomeric H2A·H2B (see the text) was also observed (fractions a, b). For additional details, see legends to Figures 3 and 9.

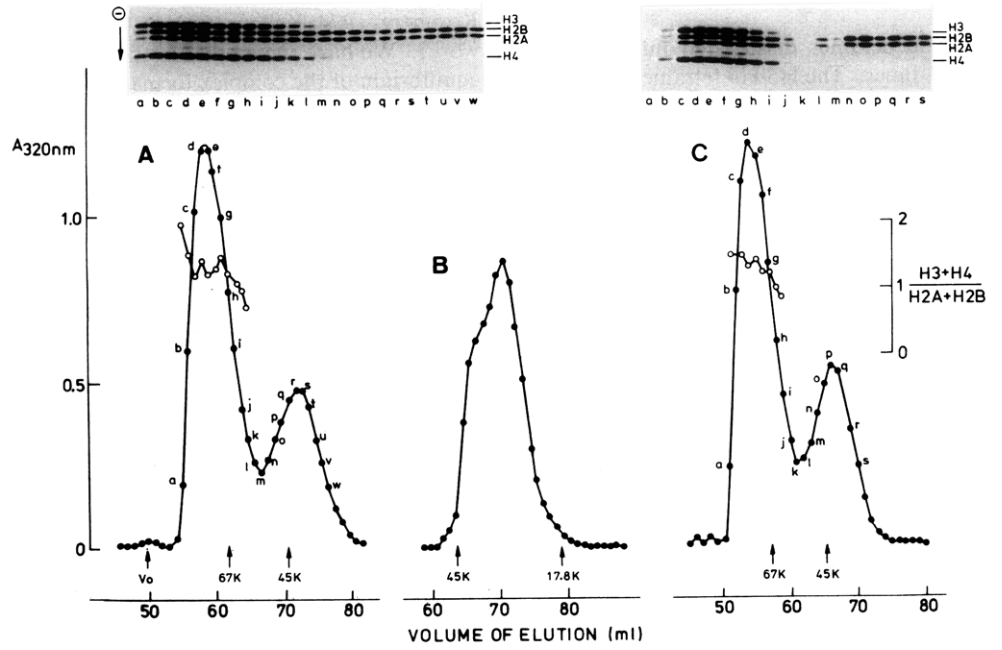

FIGURE 9: Reversibility of the core histone complex formation. The reversibility of the core histone self-assembly was examined by gel filtration chromatography in Bio-Gel P-200. (A) An aliquot of core histones in 2 M NaCl (buffer B, pH 7.2) was analyzed. (B) Elution profile of core histones as in A after 24 h of dialysis against 0.4 M NaCl (buffer B, pH 7.2). (C) Elution profile of core histones as in B after 24 h of dialysis against 2 M NaCl (buffer B, pH 7.2). Approximately 0.75 mg of histones in 0.5 mL was loaded in each case. The M_{app} of the complex in peak I in A and C was 95 000. Histones were analyzed by electrophoresis in 15–19% $NaDodSO_4$ polyacrylamide gels (inset) as indicated. V_0 was determined with [^{3}H]ferritin. For further details see legend to Figure 2.

that protonation of hystidil residues may, in part, be responsible for the destabilization of the complex.

The finding of the H3·H4 tetramer and the H2A·H2B dimer as the final products of the octamer dissociation under a variety

of conditions strongly suggests that both complexes are its basic subunits. These subunits and the type of intermediate complexes found in the octamer self-assembly suggest that the H3·H4 tetramer may have two equivalent binding domains for the H2A·H2B dimer, and, therefore, we propose that the octamer has a structure [(H2B·H2A)(H3·H4)$_2$(H2B·H2A)]. A structure with this subunit composition can have a symmetric structure originating in the H3·H4 tetramer. If the octamer reported here and that in the nucleosome share the same central structural characteristics (Thomas et al., 1977), the symmetry we propose may be related to the pseudodyad axis recently revealed in the crystal structure of the nucleosome core particle (Finch et al., 1977).

The results presented show evidence neither for the assembly of the core histone in "heterotypic" tetramers (Weintraub et al., 1975; Campbell & Cotter, 1976; Wooley et al., 1977) nor of its existence as intermediate products of the octamer dissociation (Chung et al., 1978). This and the reconstitution of the nucleosome core particle by the sequential binding of H3·H4 tetramer and H2A·H2B dimer (Ruiz-Carrillo & Jorcano, 1977, 1979) cast doubts on the existence and possible function of "heterotypic" tetramers in the chromatin. Our results support the view that the structural information for the octamer self-assembly resides in the histones and not in the DNA and that the H3·H4 tetramer can direct the organization of the histone octamer both in the absence and in the presence of DNA.

After submission of this manuscript, Chung et al. (1978) reported, on the basis of hydrodynamic studies, that the core histone complex is in equilibrium between a tetramer [*assumed* to be the "heterotypic" of Weintraub et al. (1975)] and an octamer. The finding of an octamer is essentially in agreement with our observations and those of Thomas & Butler (1977). The octamer described by Chung et al. (1978) appears to be much more dependent on protein concentration than the one we have characterized. However, in relation to this, it is worthwhile to note that the cross-linking and the hydrodynamic data presented by these authors (Chung et al., 1978) appear to be in contradiction. Thus, in a protein concentration range in which the tetramer was calculated to be the main species, the main cross-linked product was an octamer with no sign of a stop at tetrameric products. In addition, alternative ways have to be used in the characterization of the size and subunit composition of the complexes. The latter is crucial in determining the intermediate products of dissociation and in distinguishing two tetramers (i.e., H3·H4 or "heterotypic") which have similar molecular weights. Other sources of discrepancy among different laboratories may be due to (i) protein concentration and therefore $\bar{\nu}$ determination, (ii) the instability of the octamer under certain conditions, and (iii) the stoichiometry of the extracted histones. Concerning the last point, an excess of H2A·H2B in the core histone sample may average down the molecular weight obtained for the complex. More importantly, if a disproportion between H3 and H4 exists, the excess histone usually aggregates with variable amounts of other histones (A. Ruiz-Carrillo and J. L. Jorcano, unpublished observations). Unbalances of this sort may perhaps be the reason for the reported insolubility of the H3·H4 tetramer in 2 M NaCl (Weintraub et al., 1975; Chung et al., 1978) or the formation of aggregates accompanying partial dissociation of the core histone complex (Chung et al., 1978).

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H3·H4 Tetramer Directs DNA and Core Histone Octamer Assembly in the Nucleosome Core Particle†

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ABSTRACT: The way in which histones interact with DNA during in vitro assembly of nucleohistone has been examined. Chicken erythrocyte core histones H2A, H2B, H3, and H4 and λDNA in 2 M NaCl were allowed to interact by stepwise decrease in the salt concentration. Binding, although weak, was first observed at 1.4 M NaCl and was essentially completed at 0.6 M NaCl. Analysis of the DNA-bound histones revealed that each of the histones in the pairs H2A,H2B and H3,H4 was always present in equimolar amounts and that the relative proportion of each pair was constant between 1.4 and 0.8 M NaCl. Evidence is presented suggesting that binding occurred via complexes of the four histones, the nature of which is likely to reflect the equilibrium among the octamer and its products of dissociation (Ruiz-Carrillo, A., & Jorcano, J. L. (1979) *Biochemistry* (preceding paper in this issue)). The presence of complexes of the four core histones is, however, not required for the correct assembly of the nucleosome core particle. Nucleohistones obtained by adding at progressively lower ionic strengths the dimer H2A·H2B to the H3·H4–DNA complex (split reconstitutions) had the same characteristics as those assembled with the core histone complexes.

Recent work has demonstrated that core histones (H2A, H2B, H3, and H4) can form an octameric complex in solutions of high ionic strength and neutral pH (Thomas & Butler, 1977; Ruiz-Carrillo & Jorcano, 1979) and that the H3·H4 tetramer plays a central role in its organization (Ruiz-Carrillo & Jorcano, 1979). The existence of a histone octamer in the absence of DNA strongly suggests that the information for the nucleosome core particle assembly may reside in the structure of the histone complex. Therefore, if the structure of the histone octamer in solution and in the nucleosome is similar (Thomas et al., 1977), it could be expected that the octamer would bind to DNA and thus generate nucleosome-like structures. Alternatively, the preassembled histone octamer may not be necessary if the H3·H4 tetramer bound to DNA can also direct the assembly of the octamer and, as a consequence, the nucleosome core particle.

In the present report we examine this question and show that a complex of the four core histones does, indeed, bind to DNA and generates nucleosome core particle-like structures. However, evidence is also presented indicating that this can also be achieved by the sequential binding of H3·H4 tetramer and H2A·H2B dimer.

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Experimental Procedures

Cells, Isolation of Nuclei, and Histone Preparation. Nuclei from chicken erythrocytes and salt- or acid-extracted core histones and histone pairs were prepared as described previously (Ruiz-Carrillo & Jorcano, 1979). Histone concentrations were estimated by their absorbance at 275 nm (Ruiz-Carrillo & Jorcano, 1979).

Warburg ascites cells (a kind gift of Dr. E. Liss) were propagated in female NMRI mice.

Radioactive Labeling of Histones. Reductive methylation of chicken erythrocyte core histones in 2 M NaCl, 0.2 mM PMSF,[1] 10 mM sodium borate, pH 9.0, was carried out by a modification of the procedure of Rice & Means (1971) as described by Ruiz-Carrillo et al. (1975). Methylated histones

[1] Abbreviations used: b.p., DNA base pair; nucleosome core particle, 145 b.p. of DNA complexed with two of each of the core histones; core histones, H2A, H2B, H3, and H4; DNA (I), superhelical form of covalently closed circular DNA; DNA (II), relaxed form of covalently closed circular DNA; NCE, nicking and closing extract; buffer A, 5 mM Tris-HCl, 0.01% 2-mercaptoethanol, 0.1–0.3 mM PMSF, pH 7.2; buffer B, 10 mM Tris-HCl, 0.2 mM EDTA, 5% glycerol, pH 7.9; buffer C, 5 mM Tris-HCl, 0.1 mM EDTA, 0.01% 2-mercaptoethanol, 0.1 mM PMSF, pH 7.2; PMSF, phenylmethylsulfonyl fluoride; SSC, 0.15 M NaCl, 0.015 M sodium citrate; EDTA, sodium salt of ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; TBE, 90 mM Tris-boric acid, 2.5 mM EDTA, pH 8.3.

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