

The Histone Core Complex: An Octamer Assembled by Two Sets of Protein-Protein Interactions[†]

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ABSTRACT: A protein complex, extracted from calf thymus chromatin with 2 M NaCl, pH 7.5, containing approximately equal molar ratios of histones H2A, H2B, H3, and H4, has been characterized in this study. Gel filtration, sedimentation velocity, and sedimentation equilibrium experiments demonstrate that this complex, known as the core complex, has a molecular weight near that expected for a histone octamer (108 000 for a unit containing two each of the four inner histones) and far exceeding that of a histone tetramer (54 400). This finding suggests that the histone octamer, postulated to be the fundamental histone unit in chromatin, is stable in 2 M NaCl, pH 7.5, in the absence of DNA or chemical cross-linking reagents. In the second part of this study, we demonstrate that the bonds maintaining the octameric complex in 2 M NaCl are weak and distinctly different from the forces stabilizing the

H2A-H2B dimer or H3-H4 tetramer. The octamer is dissociated into two H2A-H2B dimers and one H3-H4 tetramer by (i) increasing temperature; (ii) decreasing NaCl concentration; (iii) adding low concentrations of urea or guanidine hydrochloride; and (iv) lowering the pH below 7 or raising it above 10. These findings indicate that the octamer is assembled by two sets of protein-protein interactions. The first set involves mostly hydrophobic interactions and yields the H2A-H2B dimer and the H3-H4 tetramer subunits. The second set involves the weak association of one H3-H4 tetramer with two H2A-H2B dimers to form an octamer. We suggest that these weak interactions might be derived predominantly from histidine-lysine or histidine-tyrosine hydrogen bonds between the dimer and tetramer subunits.

Much of the current research in the field of chromatin structure is based on the discovery that the histone components of the complex are arranged along the DNA backbone in a "repeated array." It is generally accepted that this periodic arrangement of histones along the DNA gives rise to the nucleosomes obtained after nuclease digestion or the "beads" seen during electron microscopy of chromatin (Hewish & Burgoyne, 1973; Rill & Van Holde, 1973; Woodcock, 1973; Olins & Olins, 1974; Noll, 1974). Kornberg (1974) first suggested that this repeated array involved two each of the four core histones (H2A, H2B, H3, and H4) and one molecule of H1 for every 200 base pairs of DNA. As evidence that the core histones do exist in chromatin as an octamer, Thomas & Kornberg (1975) demonstrated that a protein complex, which had a molecular weight near that expected for a histone octamer, could be isolated from chromatin previously treated at pH 9 with diimido ester cross-linking reagents. They further demonstrated that this octamer could be obtained if cross-linking of the chromatin was conducted in 2 M NaCl, an environment in which the histones are released from the DNA. At about the same time, Weintraub et al. (1975) described a non-cross-linked histone complex, isolated in 2 M NaCl, pH 7, which contained equal molar ratios of the four core histones. The molecular weight they determined for the complex was near that expected for a histone tetramer. They suggested that each tetramer, termed "heterotypic tetramer," contained one each of the four core histones, and that two of these tetramers would join together to form an octamer when bound to DNA. Since these initial observations, additional studies have been presented in support of a tetramer model for the core histones in 2 M NaCl (Campbell & Cotter, 1976; Wooley et al., 1977; Pardon et al., 1977), a heterotypic tetramer in equilibrium with

an octamer (Chung et al., 1978), as well as an octamer model (Thomas & Butler, 1977).

In this report, we demonstrate by gel filtration, sedimentation velocity, and sedimentation equilibrium experiments that the histone core complex exists in 2 M NaCl, pH 7.5, as an octamer. DNA and/or protein cross-linking reagents are not required for the formation of this complex. Additional evidence is presented which leads us to propose that this histone complex is maintained as an octamer by hydrogen bonds between one H3-H4 tetramer and two H2A-H2B dimer subunits.

Materials and Methods

Chromatin Isolation and Histone Extraction. Calf thymus chromatin was isolated essentially as described earlier (Rubin & Moudrianakis, 1972; 1975). Calf thymus tissue was homogenized in 85 mM NaCl, 50 mM NaHSO₃, 3 mM MgCl₂, pH 7.5, and filtered successively through multiple layers of cheesecloth and one layer of miracloth. Nuclei were pelleted by low-speed centrifugation and repeatedly washed in homogenization buffer until the supernatant was clear. Swelling and rupture of the nuclei were accomplished by successive washings first in 50 mM NaHSO₃, pH 7.5, then in 10 mM NaHSO₃, 1 mM EDTA, pH 7.5, and finally in distilled water. The resultant gel was made 3 mM EDTA, pH 7.5, concentrated by low-speed centrifugation, and sheared in a Waring blender at 80 V for 10 s. Histone complexes were extracted from the isolated chromatin (2 mg/mL) by the addition of an equal volume of either 4 M NaCl, 20 mM Tris-HCl, pH 7.5, 2 mM phenylmethanesulfonyl fluoride, or 4 M NaCl, 20 mM NaH₂PO₄, pH 5.0. DNA was separated from the dissociated histone by centrifugation at 44 000 rpm in a Ti50 rotor. All operations were performed at 4°C.

Chromatin was prepared from the mature erythrocytes of 30-day-old chicks (Truslow Farms, Inc.) according to the procedure of Anderson (1971). Briefly, this technique involves collecting the erythrocytes in 0.1 M NaCl, filtration through miracloth, and repeated washings in 0.01 M NaCl. Cell lysis

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was achieved by glass-Teflon homogenization in 0.01 M NaCl, 10 mM Tris-HCl, pH 7.5. Nuclei were purified by consecutive centrifugations through 1.2 M sucrose and 1.8 M sucrose, followed by extraction with 0.08 M NaCl, 0.3 M NaCl, 0.3 M sucrose, 20 mM EDTA, pH 7.5. Further swelling and rupture of the purified nuclei were accomplished by repeated washings in decreasing molarities of Tris-HCl buffer. Isolation of histone from the resultant gel was accomplished as described for calf thymus chromatin.

Gel Filtration. Histone complexes were fractionated on a Sephadex G-100 column (2.5 × 155 cm) equilibrated in 2 M NaCl, 10 mM Tris-HCl, pH 7.5, or in 2 M NaCl, 10 mM NaH₂PO₄, pH 5.0. Protein supernates from the 2 M NaCl extracts of calf thymus chromatin (approximately 80 mg of total protein in 95 mL) were concentrated to 10 mL by vacuum dialysis in 0.25-in. dialysis tubing (Arthur H. Thomas Co.) and loaded onto the G-100 column. Six-milliliter fractions were collected at a flow rate of 16 mL/h. The core complex was purified by pooling the area under its peak and again concentrating the protein by vacuum dialysis. The purified core complex could be stored at 4 °C for several weeks without dissociating or showing any signs of proteolytic degradation.

The following proteins were selected and used as standards in the determination of the apparent molecular weight of the core complex: cytochrome *c* (horse heart), 13 500; myoglobin (horse skeletal muscle), 17 000; ovalbumin, 44 000; serum albumin (bovine), 65 000; serum albumin dimer (bovine), 130 000. Each protein was dissolved in 10 mM Tris-HCl, pH 7.5, and dialyzed against 2 M NaCl, 10 mM Tris-HCl, pH 7.5, before being loaded onto a G-100 column.

Sucrose Gradient Sedimentation. The histone core complex and the various protein standards were dialyzed against 2 M NaCl, 10 mM Tris-HCl, pH 7.5. Sample volumes of 0.2 mL were layered onto 5–20% (w/v) sucrose gradients containing 2 M NaCl, 10 mM Tris-HCl, pH 7.5, and centrifuged at 4 °C in a SW 50.1 rotor at 45 000 rpm for 48 h. Proteins used as molecular weight standards were identical with those in the gel filtration experiments.

Equilibrium Sedimentation. All experiments were conducted with G-100 purified core complex which had been extensively dialyzed against 2 M NaCl, 10 mM Tris-HCl, pH 7.5. High-speed sedimentation equilibrium experiments were performed using a Beckman Model E analytical ultracentrifuge equipped with a Rayleigh interference optical system. A complete description of the experimental procedures followed can be found in the study of Godfrey & Harrington (1970b). Weight average molecular weights were determined according to the method of Yphantis (1964), using the computer program developed by Roark & Yphantis (1969). The partial volume, ϕ' , of the core complex was determined by the method of Cassassa & Eisenberg (1964) for multicomponent systems at dialysis equilibrium. The quantity termed ϕ' by these authors yields true molecular weights of proteins which strongly bind solvent components (see Eisenberg, 1976). It was assumed that the partial volume of the core complex was concentration independent. Measurement of the density increment was obtained at 20 °C in 2 M NaCl, 10 mM Tris-HCl, pH 7.5, with a DMA-02 C frequency densitometer (Anton Paar K. G., Austria). Protein concentrations were determined with the biuret protein assay as described by Zamenhoff (1957). A stock solution of core histone from which a known volume had been removed for a nitrogen determination (Galbraith Laboratories, Inc.) was used as the color standard in this protein assay. In three separate determinations, and using three different preparations of the purified core complex at concen-

trations from 3.9 to 7.8 mg/mL, the partial volume was found to be 0.753 ± 0.003 cm³/g.

Determination of the partial volume of the core complex and all sedimentation equilibrium measurements were conducted in the laboratory of Dr. William F. Harrington of this department.

Small-Zone Exclusion Chromatography Studies. The association properties of the purified core complex were analyzed by the small-zone exclusion chromatography technique (Ackers, 1970) using Sephadex G-100 columns (1.7 × 115 cm) maintained at flow rates of 8–10 mL/h. For the temperature stability studies, 2-mL aliquots of the sample solutions were allowed to equilibrate at the new temperature for 2 h before the columns were loaded. In the NaCl, urea, guanidine hydrochloride, and pH stability studies, the 2-mL aliquots were dialyzed at 4 °C for at least 5 h against 500 mL of the appropriate solvent. After this dialysis, protein concentrations were determined and equal amounts of protein were loaded onto each column. For accurate comparison of the eluted volumes of the various histone complexes, the columns were frequently calibrated using bovine serum albumin and cytochrome *c* as standards.

Gel Electrophoresis. The histone content of the various histone complexes was characterized on 10% polyacrylamide/NaDodSO₄¹-urea gels as previously described (Eickbush et al., 1976). All samples were desalted and lyophilized before incubation with the NaDodSO₄-urea loading buffer.

Results

Histone Complexes Extracted from Chromatin with 2 M NaCl. When calf thymus histones are extracted from chromatin with 2 M NaCl, 10 mM NaH₂PO₄, pH 5, and applied to a Sephadex G-100 column equilibrated with the same buffer, they yield the elution profile shown in Figure 1a. This protein elution profile is similar to the G-100 profile originally obtained by Van der Westhuyzen & von Holt (1971). Analysis of the proteins under each peak by NaDodSO₄-polyacrylamide gel electrophoresis indicates that the first major peak is composed of histones H1, H3, and H4 (gel A). The arginine-rich histones of this peak in the Van der Westhuyzen & von Holt profile have previously been characterized to exist as the tetrameric complex (H3-H4)₂ (Kornberg & Thomas, 1974; Roark et al., 1974; Moss et al., 1976b). The second major peak contains histones H2A and H2B (gel B). These two histones have been demonstrated to be eluted from the Van der Westhuyzen & von Holt column as the dimer H2A-H2B (Moss et al., 1976a). The small adsorption peak which is found with the excluded volume of the column (227 mL) contains most of the nucleic acids and nonhistone proteins which are contaminating this type of extract.

The elution profile of the histone complexes extracted from calf thymus chromatin with 2 M NaCl, 10 mM Tris-HCl, but this time at pH 7.5, and fractionated on a G-100 column equilibrated with the same buffer, is shown in Figure 1b. The proteolytic inhibitor phenylmethanesulfonyl fluoride (Fahrney & Gold, 1963) has been included in the extraction buffer, since the extraction of calf thymus chromatin with 2 M NaCl at a pH greater than 5.5 results in the proteolytic cleavage of H2A (Eickbush et al., 1976). This cleavage of H2A has a dramatic effect on the formation of histone complexes, and will be the subject of a separate report (Eickbush & Moudrianakis, in preparation).

¹ Abbreviations used: NaDodSO₄, sodium dodecyl sulfate; BSA, bovine serum albumin.

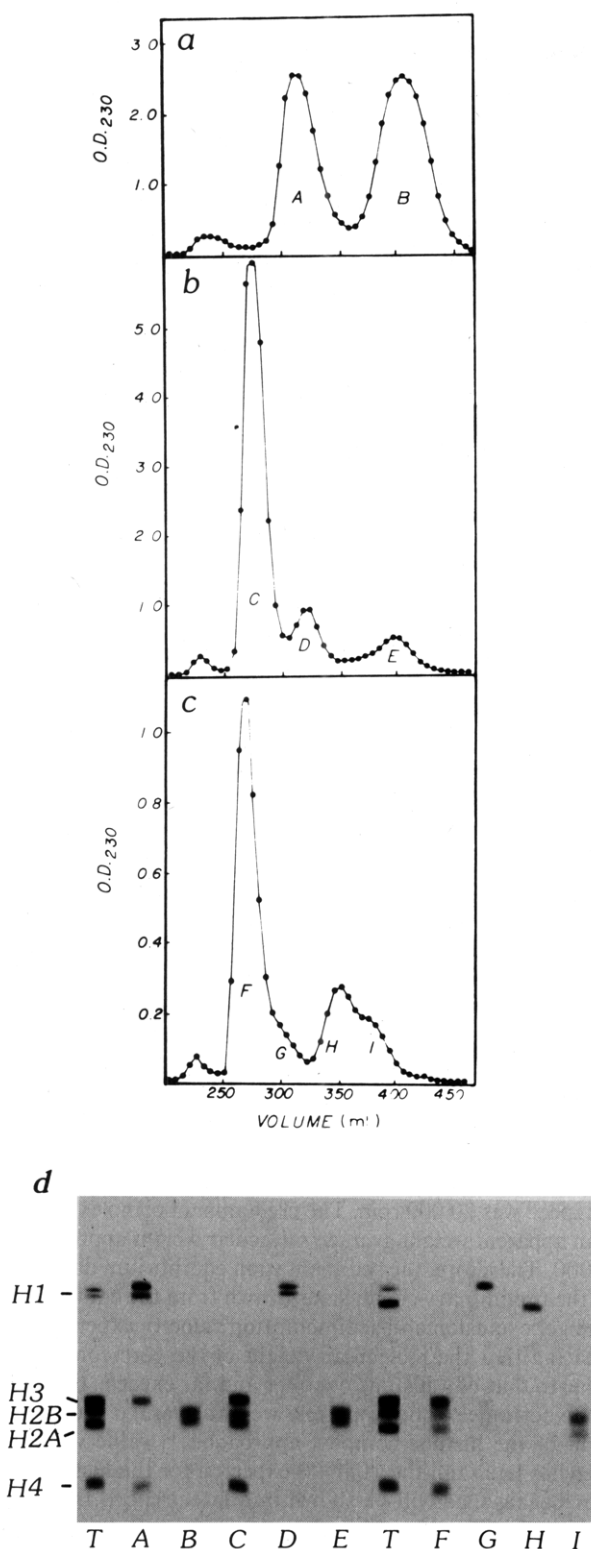


FIGURE 1: Sephadex G-100 fractionation of histone complexes extracted from chromatin with 2 M NaCl. DNA was pelleted by ultracentrifugation and the supernatant concentrated by vacuum dialysis before application to the column. (a) Histone complexes extracted from calf thymus chromatin with 2 M NaCl, 10 mM NaH_2PO_4 , pH 5, and eluted from the column with the same buffer. (b) Histone complexes extracted from calf thymus chromatin with 2 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM phenylmethanesulfonyl fluoride, and eluted from the column with 2 M NaCl, 10 mM Tris-HCl, pH 7.5. (c) Histone complexes extracted from chick erythrocyte chromatin with 2 M NaCl, 10 mM Tris-HCl, pH 7.5, and eluted from the column with the same buffer. (d) NaDodSO₄-urea polyacrylamide gels of the proteins isolated from each peak (marked A through I) in profiles a-c. Gels marked T contain the total histone of thymus or erythrocytes.

It is apparent from a comparison of Figures 1a and 1b that the histone interactions which occur in 2 M NaCl, pH 7.5, are different from those which occur in 2 M NaCl, pH 5. At pH 7.5, all the H3 and H4 and the majority of H2A and H2B are associated into a complex, which we shall call the core complex, eluting ahead of the tetramer region of the profile (gel C). The percentage of the H2A and H2B eluted from the column in the dimer position (gel E) is dependent upon the concentration of protein initially loaded onto the column. Increasing loading concentrations result in a decrease in the percentage of protein in the core complex peak. As described in greater detail below, this finding suggests that the H2A-H2B dimer is in equilibrium with the histones in the core complex peak. In all subsequent experiments, where we have purified the core complex, we have consistently loaded onto the column a high concentration of protein (80 mg). This concentration results in the migration of 25% of the H2A and H2B through the column as a dimer, while the core complex peak is correspondingly enriched in the H3 and H4 histones, as determined by NaDodSO₄-polyacrylamide electrophoresis. Indeed, the excess H3 and H4 found in this peak is approximately equal to the amount of H2A and H2B found in the dimer region of the profile. Thus equal molar ratios of the four core histones were applied to the G-100 column, in agreement with the evidence suggesting that these histones are present in chromatin in equal numbers (Joffe et al., 1977). Trace amounts (<2% of the total protein) of an approximately 25 000 molecular weight, unique, nonhistone protein also can be found in the core protein peak. We are currently investigating whether this protein is associated directly with the histone complex.

Weintraub et al. (1975) have reported a pH dependence in the formation of histone complexes isolated by 2 M NaCl extraction of chick erythrocyte chromatin. They demonstrated, by a variety of techniques, that the H2A-H2B dimer and the H3-H4 tetramer remain separate species in 2 M NaCl at pH 5.5, while a complex containing all four histones could be isolated in 2 M NaCl at pH 7.1. This complex eluted from a Bio-Gel A-0.5 column as a broad peak slightly after the H3-H4 tetramer. As can be seen in Figure 1b, the histone complex we extract from calf thymus chromatin, which contains all four core histones, is eluted from the G-100 column as a sharp peak significantly before the H3-H4 tetramer. To determine if this difference in the elution properties of the two core complexes is a result of tissue or species differences, we have also isolated the complex from chick erythrocyte chromatin, Figure 1c. The core complex extracted from chick erythrocyte chromatin migrates in a manner identical with that isolated from calf thymus. The only difference between the calf thymus profile and the chick erythrocyte profile is the presence in the latter, of the erythrocyte-specific histone, H5. This protein migrates on the column slightly ahead of the H2A-H2B dimer peak (gels H and I, Figure 1d).

Molecular Weight of the Core Complex. The core complex, isolated from a G-100 column as in Figure 1b, is stable at 4 °C in 2 M NaCl, pH 7.5. No histone aggregation or irreversible dissociation of the complex can be seen when the purified core complex is reloaded from the same G-100 column (Figure 2a) or analyzed on sucrose gradients (Figure 2b). Two characteristics of the core complex are apparent from these elution and sedimentation profiles. First, the leading edge of the solute zone is sharper than the trailing edge. This trailing is not due to the presence of contaminating, lower molecular weight complexes. If the leading edge of the solute zone is pooled, concentrated, and reloaded to a G-100 column or sucrose gradient, the shape of the solute zone in the second purification run is not changed. As will be described in greater detail below,

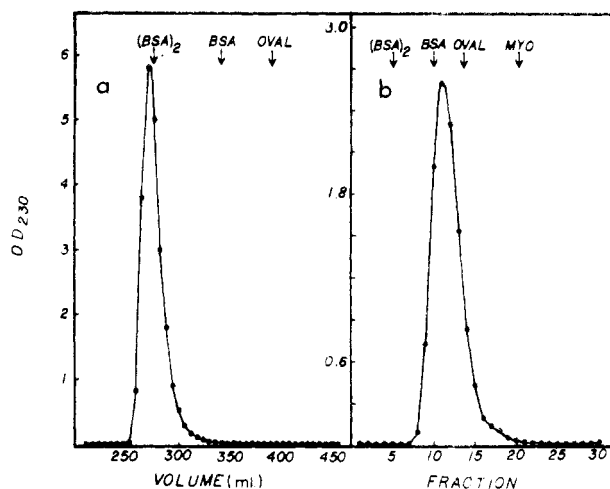


FIGURE 2: Apparent molecular weight of the histone core complex determined by gel filtration and sedimentation velocity. The core protein peak from a G-100 profile similar to that in Figure 1(b) was pooled, concentrated, and stored for 1 week at 4 °C. (a) Elution profile of the purified complex reeluted from the same G-100 column as in Figure 1 with 2 M NaCl, 10 mM Tris-HCl, pH 7.5. (b) Centrifugation profile of the purified core complex on 5–20% sucrose gradients also in 2 M NaCl, 10 mM Tris-HCl, pH 7.5. Centrifugation was conducted in a SW 50.1 rotor for 48 h at 4 °C and 45 000 rpm. Direction of migration was from right to left. Peak positions of the protein standards are indicated by the arrows. Protein standards shown are: (MYO) myoglobin, 17 000 daltons; (OVAL) ovalbumin, 44 000 daltons; (BSA) bovine serum albumin, 65 000 daltons; (BSA)₂ bovine serum albumin dimer, 130 000 daltons.

the core protein elution profile is characteristic of a complex which is undergoing rapid, reversible association (Winzor & Scheraga, 1962).

Second, as can be seen in Figure 2, the apparent molecular weight of the core complex determined by gel filtration is radically different from the apparent molecular weight determined by sedimentation velocity. Compared with various globular protein standards, the complex is eluted from the G-100 column as if its molecular weight were 135 000, while the complex sediments through a sucrose gradient as if its molecular weight were only 55 000. The radically different behavior exhibited by the core complex in these two transport methods implies that the complex is either nonglobular in shape and/or has a very high partial specific volume (Martin & Ames, 1961; Siegel & Monty, 1966). As will be discussed in more detail below, the partial volume of the core complex in 2 M NaCl is indeed higher than the value normally assumed for proteins. However, this density difference will result in only a 20% increase in the apparent molecular weight of the core complex determined on a sucrose gradient. One can therefore conclude that the core complex is nonglobular; in other words, the complex has a molecular radius which is larger than that expected for a globular protein of similar molecular weight.

These gel filtration and sedimentation velocity experiments clearly suggest that the molecular weight of the core complex is significantly greater than 55 000, while less than 135 000. We can therefore identify the molecular weight of this histone complex, containing approximately equal molar ratios of the core histones, as being similar to that of an octamer (108 800 for a unit containing two each of the four histones), while exceeding that of a tetramer (54 400 for a unit containing only one of each histone). However, the atypical behavior of the core complex in these transport methods suggests that any molecular weight determination based solely upon these two methods should be regarded as somewhat tentative. We have therefore determined the molecular weight of the complex by the sedimentation equilibrium technique, since once the partial volume

of a protein is known, its molecular weight can be accurately determined regardless of its shape.

The partial specific volume, \bar{v} , for various histone complexes has been reported to be in the range of 0.725 to 0.733 cm³/g (Roark et al., 1974; 1976; D'Anna & Isenberg, 1974; Weintraub et al., 1975). These values were calculated from the amino acid composition of the histones using the density for individual amino acids originally reported by Cohn & Edsall (1943). While obviously it is always best to experimentally determine the partial specific volume of a protein, the need for a direct determination is even more important in the case of the core histones, since our sedimentation equilibrium experiments are done in 2 M NaCl. At high ionic strengths, preferential binding of solvent components to protein can lead to significant changes in their partial volumes (Godfrey & Harrington, 1970a). We have therefore determined the partial volume, ϕ' , of the core histones (rather than the partial specific volume, \bar{v}), a quantity which yields true molecular weight values even if the protein strongly binds solvent components (Eisenberg, 1976). In three separate determinations using three preparations of the purified core complex in 2 M NaCl, 10 mM Tris-HCl, pH 7.5, at concentrations ranging from 3.9 to 7.8 mg/mL, the partial volume was determined to be 0.753 ± 0.003 cm³/g. This high value for the partial volume suggests that in 2 M NaCl, pH 7.5, there is preferential binding of water to the protein complex. Thomas & Butler (1977) have reported the partial volume ϕ' of the core histones in 2 M NaCl to be 0.767 cm³/g. Their measurement of the density increment and calculation of the partial volume were conducted by the same methods we have used. We believe the major reason for the different values obtained could be that Thomas & Butler determined the protein concentration by amino acid analysis after acid hydrolysis, while we have estimated the protein concentration by total nitrogen determination.

High-speed sedimentation equilibrium experiments were performed according to the methods of Yphantis (1964), and the data analyzed using the computer program of Roark & Yphantis (1969). Figure 3a presents results of the sedimentation equilibrium experiments with the G-100 purified core complex in 2 M NaCl, pH 7.5, at a loading concentration of 1 mg/mL. The temperature was maintained at 4 °C, and the rotor speed was 20 000 rpm. The predominant complex present has an apparent weight average molecular weight approaching 100 000. Therefore, the sedimentation equilibrium data support the preliminary conclusions drawn from the comparison of the gel exclusion and sedimentation velocity experiments, indicating that the molecular weight of the core complex is similar to that of a histone octamer and far exceeds that of a histone tetramer. The apparent weight average molecular weight of the histone complex approaches a value which is somewhat less than the 108 000 expected for the histone octamer because, as will be shown in a later report, the associating system has a positive virial coefficient. Detailed equilibrium sedimentation experiments to determine the histone complexes involved in the formation of the octamer and their association constants will be the subject of a separate communication (Godfrey, Eickbush, & Moudrianakis, in preparation).

Temperature Effects on the Core Complex. Both the trailing of the solute peaks shown in Figure 2 and the upward trend in the weight average molecular weight determinations described above were conducted at 4 °C. We now describe the effects of increasing temperature on the association of the core complex.

Sedimentation Equilibrium. Figure 3b presents results of sedimentation equilibrium experiments with the purified core

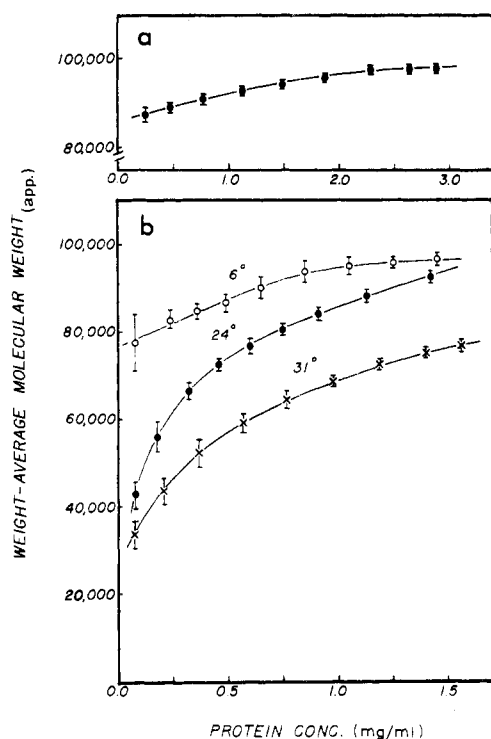


FIGURE 3: High-speed sedimentation equilibrium experiments of the purified core complex in 2 M NaCl, 10 mM Tris-HCl, pH 7.5. (a) Determination of the apparent weight average molecular weight. The experiment was conducted at 4 °C, the initial loading concentration was 1 mg/mL, and the rotor speed 20 000 rpm. (b) Determination of the effects of temperature upon the apparent weight average molecular weight. At each temperature the initial loading concentration was 2 mg/mL and the rotor speed 30 000 rpm: (○) 6 °C; (●) 24 °C; (+) 31 °C.

complex in 2 M NaCl, pH 7.5, at three different temperatures. At each temperature the protein loading concentration was 2 mg/mL and the rotor speed was 30 000 rpm. Dissociation of the octamer at the lower protein concentrations in the cell is more extensive at 24 °C than at 6 °C. At both temperatures, however, the apparent weight average molecular weight of the maximally associated complex is near that of the histone octamer. This suggests that, while the association constant of the core complex decreases with increasing temperature, the octamer still is the predominant species present at 24 °C at protein concentrations above 1 mg/mL. At 31 °C, the weight average molecular weight of the core complex is dramatically reduced throughout the centrifuge cell, approaching 80 000 at the higher protein concentrations. The octamer, even at high protein concentrations, is no longer the predominant protein complex found in solution at 31 °C. Equilibrium sedimentation experiments at temperatures greater than 31 °C cannot be reproducibly conducted in the analytical ultracentrifuge due to the spread of oil vapors from the vacuum system.

Gel Filtration. As shown in Figure 2a, when the purified core complex is subjected to this analysis, the leading edge of the solute zone appears hypersharp while there is boundary spreading of the trailing edge. This is the anticipated behavior of a reversibly associating system since any experimental condition which leads to a decrease in the association constant of the complex should result in an increase in the amount of protein eluted at the trailing edge of the solute peak (Winzor & Scheraga, 1963; Ackers, 1970). This phenomenon enables one to use exclusion chromatography to determine relative stabilities of protein complexes.

An advantage this technique offers over sedimentation equilibrium is that the subunits into which the complex dis-

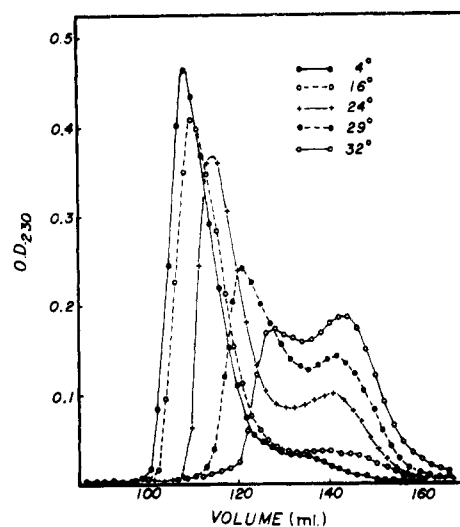


FIGURE 4: Small-zone gel filtration technique to determine octamer stability as a function of temperature. Each profile represents the elution of 2 mL of the purified octamer (1 mg/mL) on G-100 columns equilibrated at the indicated temperatures in 2 M NaCl, 10 mM Tris-HCl, pH 7.5.

sociates (e.g., the H2A-H2B dimers and H3-H4 tetramer in Figure 4) can be directly isolated and analyzed. It is important to remember, however, that due to the concentration-dependent behavior of associating systems at any given temperature, the ratio between the various components in the system will vary with the concentration of protein initially loaded on the column.

Small G-100 columns were used for these stability studies, since they require less time and protein than the large preparative columns used in Figure 1, yet still provide clear resolution of the various histone complexes. Figure 4 presents the elution profiles of the purified core complex in 2 M NaCl, pH 7.5, at several temperatures. At 4 and 16 °C sharp octamer peaks are obtained with only minimal amounts of protein trailing. When assayed by NaDodSO₄-polyacrylamide gel electrophoresis this trailing protein is comprised of equal amounts of H2A and H2B (gels not shown), corresponding to the loss of H2A-H2B dimer from the core complex. This is the expected behavior for a reversibly associating system responding to a decrease in the amount of protein applied on the column (compare profiles to Figure 2a). At 24 °C a distinct trailing H2A-H2B dimer peak is found, while the position of the former octamer peak has shifted to the right (in the direction of lower molecular weight) and is now highly enriched in H3 and H4 as assayed on NaDodSO₄-polyacrylamide gels. This gradual loss of H2A and H2B from the octamer peak and the corresponding shift in the apparent molecular weight of this peak to lower values continues with increasing temperatures until, at 32 °C, a bimodal peak comprising the H3-H4 tetramer and the H2A-H2B dimer species is eluted from the column. At temperatures greater than 32 °C aggregation of the H3-H4 tetramer results in the loss of H3 and H4 from the tetramer region of the profile. These gel filtration results are in complete agreement with the equilibrium sedimentation experiments; the association constant of the histone octamer decreases as the temperature increases.

It can also be seen from Figure 4 that at 24 °C a majority of the protein is eluted from the column at a position intermediate between that of a histone octamer and that of a tetramer. The molar ratios of the core histones in this peak correspond to those of a hexamer composed of one H3-H4 tetramer and one H2A-H2B dimer. Thus we believe such a hexamer complex is a predominant component of this inter-

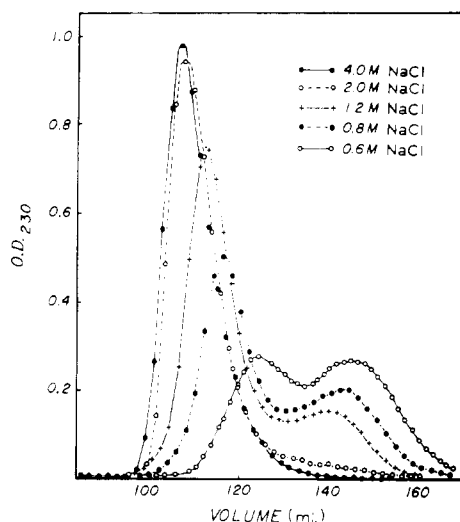


FIGURE 5: Small-zone gel filtration technique to determine octamer stability as a function of NaCl concentration. Each profile represents the elution of 2 mL of the purified octamer (2 mg/mL) on G-100 columns equilibrated at 4 °C at the indicated NaCl concentrations.

mediate elution peak. In a subsequent report, equilibrium sedimentation data will be presented which also suggest the presence of an intermediate hexamer species in the formation of the histone octamer (Godfrey, Eickbush, & Moudrianakis, in preparation). It should be mentioned, however, that because the system is reversibly associating, the "hexamer peak" seen in Figure 4 also contains significant amounts of complete octamers as well as H3-H4 tetramers. The protein is eluted from the column as a distinct peak, due to the boundary sharpening which occurs at the leading edge of the protein solute zones containing associating systems (Winzor & Scheraga, 1963; Ackers, 1970).

Effects of NaCl Concentration on the Core Complex. All molecular weight determinations and protein stability studies described to this point were conducted in 2 M NaCl, the concentration of NaCl in the original chromatin extraction step. Based upon tryptic digestion studies, Weintraub et al. (1975) suggested that the core complex dissociated at low concentrations of NaCl. We have directly tested the effects of lowering the concentration of NaCl upon the stability of the core complex, again using the small-zone molecular sieve chromatographic technique.

Figure 5 presents the elution profiles of the purified octamers resolved on G-100 columns equilibrated with several concentrations of NaCl. All experiments were performed at 4 °C in 10 mM Tris-HCl, pH 7.5. Increasing the NaCl concentration from 2 to 4 M leads to an increase in the association constant of the octamer, as can be deduced from the reduction in the amount of protein trailing from the octamer peak. Lowering the NaCl concentration below 2 M leads to a significant decrease in the association constant of the octamer, as evidenced by the increase in the amount of protein at the trailing edge of the solute zone. As in the case of the temperature studies, this increased trailing can be attributed to the gradual buildup of an H2A-H2B dimer peak, while the octamer peak gradually shifts in the direction of lower molecular weight until it becomes the H3-H4 tetramer. During this shift, the ratio of H2A + H2B to H3 + H4 in this peak decreases (gels not shown). By 0.6 M NaCl a double peak representing the H3-H4 tetramer and the H2A-H2B dimer is eluted from the column.

Evidence That the Core Complex is Maintained by Hydrogen Bonds. Figures 3b and 4 demonstrate that the histone octamer is dissociated by increasing temperatures. A decrease

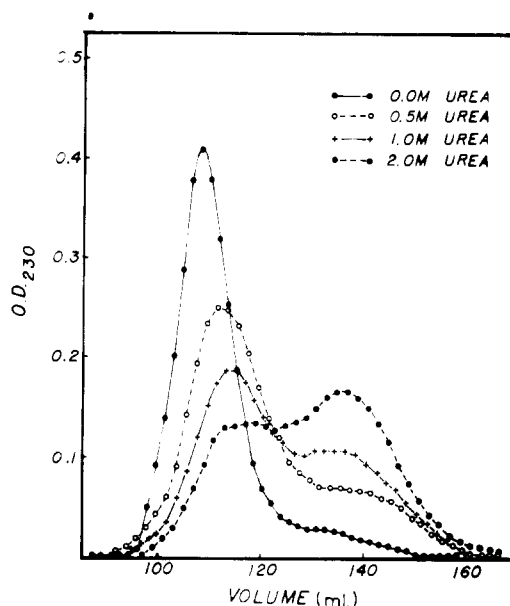


FIGURE 6: Small-zone gel filtration technique to determine octamer stability as a function of urea concentration. Each profile represents the elution of 2 mL of the purified octamer (1 mg/mL) on G-100 columns equilibrated at 4 °C in 2 M NaCl, 10 mM Tris-HCl, pH 7.5, with the indicated urea concentration.

in the stability of a protein complex with increasing temperature has been taken to indicate that the subunits of that complex self-interact predominantly via the formation of intermolecular hydrogen bonds (Scheraga, 1963). Examples of protein complexes which are believed to be stabilized by hydrogen bonds include β -lactoglobulin (Townend et al., 1960; Townend & Timasheff, 1960) and the polymerization of fibrinogen to fibrin (Sturtevant et al., 1955). In both of these systems increasing temperature results in the dissociation of the complex.

If the histones in the core complex associate through the formation of hydrogen bonds, one would expect that low concentrations of reagents known to disrupt hydrogen bonds would dissociate the octamer into its subunits. Urea was selected for this purpose because its effects upon the forces which maintain protein complexes have been most widely analyzed (Tanford, 1968, 1970). Figure 6 presents the elution profiles of the purified octamer run on G-100 columns equilibrated at 4 °C in 2 M NaCl, pH 7.5, with increasing concentrations of urea. It can be seen that 0.5 M urea has a radical destabilizing effect upon the octamer. In 2 M urea, no protein is eluted in the octamer region of the profile, and distinct H3-H4 tetramer and H2A-H2B dimer peaks are observed.

We have also tested the effects of increasing concentrations of guanidine hydrochloride upon the formation of the histone octamer. Guanidine hydrochloride at a concentration of 0.5 M resulted in the complete dissociation of the octamer on the G-100 columns, resulting once again in H3-H4 tetramers and H2A-H2B dimers (profiles not shown). Therefore, on a molar basis, guanidine hydrochloride is approximately four times as effective as urea in dissociating the histone octamer.

Since high concentrations of urea and guanidine hydrochloride can affect hydrophobic interactions as well as hydrogen bonding (Kawahara et al., 1965), we cannot conclude that the associations between the H2A-H2B dimers and H3-H4 tetramers occur exclusively by means of hydrogen bonds. On the other hand, we can conclude that the interaction between these subunits is much weaker than the interaction within the H2A-H2B dimer and H3-H4 tetramer, and that the low concentration of these reagents required to dissociate

the complex is consistent with the suggestion that the octamer is maintained primarily by hydrogen bonding between these subunits.

Hydrogen bonds between protein subunits involve the ionization of specific amino acid R groups. A study of the effects of pH upon the association of the histones may therefore indicate which R groups are involved in the hydrogen bonds maintaining the octameric complex. Figure 7a presents our results with G-100 columns run at 4 °C in 2 M NaCl and at a pH varying from 7 to 5. An extensive dissociation of octamers to tetramers and dimers occurs when the pH is lowered from 6.5 to 5.5. Since the pK of the histidine imidazole group is 6.0, the abrupt drop in octamer stability around pH 6 implicates the ionization of histidine in the formation of the histone octamer.

If the pH is raised above 7, no significant change in the stability of the octamer is noted until pH 10. At that point, as shown in Figure 7b, there is another abrupt drop in the stability of the octamer such that by pH 11 we have again returned to the H3-H4 tetramer and the H2A-H2B dimers. The ϵ -amino group of lysine amino acids has a pK of 10.5, while the pK of the phenol group of tyrosine is 10.1. We therefore suggest that the hydrogen bonds, which we have postulated on the basis of temperature and protein denaturant studies to be involved in the formation of the octamer, involve lysine or tyrosine residues as the hydrogen donor and histidine residues as the hydrogen acceptor.

Discussion

In this report we have characterized the histone core complex extracted from calf thymus chromatin with 2 M NaCl, pH 7.5. Based upon gel filtration, sedimentation velocity and sedimentation equilibrium data, we have determined the molecular weight of this complex to be approximately 100 000. This value is very close to that expected for the molecular weight of a histone octamer containing two each of the four core histones (108 800).

Conflicting conclusions have been presented concerning the aggregation state of the core histones in 2 M NaCl (Thomas & Kornberg, 1975; Weintraub et al., 1975; Campbell & Cotter, 1976; Wooley et al., 1977; Thomas & Butler, 1977; Chung et al., 1978). Postulated tissue and/or species differences in the histone complexes cannot adequately explain this controversy, since we have extracted the core histones from calf thymus and chick erythrocyte chromatin, and have found no significant differences in the molecular weight of the two types of core complexes isolated (Figure 1). Some of the factors which we believe are responsible for the conflicting molecular weight determinations reported are as follows. First, substantially different values have been assumed for the partial specific volumes of the core histones in 2 M NaCl (Weintraub et al., 1975; Thomas & Butler, 1977; Chung et al., 1978). These differences can lead to as much as a 25% variation in the molecular weight determined from equilibrium sedimentation experiments. Second, certain experiments have been conducted using low-speed equilibrium sedimentation techniques (Weintraub et al., 1975; Chung et al., 1978). As will be described in greater detail elsewhere (Godfrey, Eickbush, & Moudrianakis, in preparation), this technique fails to detect the presence of the H2A-H2B dimer species, thereby leading to the characterization of a larger percentage of the protein as a tetramer. Third, it is not clear that all experiments have been conducted at sufficiently low temperatures (Campbell & Cotter, 1976). The ability of increasing temperatures to dissociate the core complex is clearly demonstrated in Figures 3b and 4 of the present report. Fourth, and what we believe to be

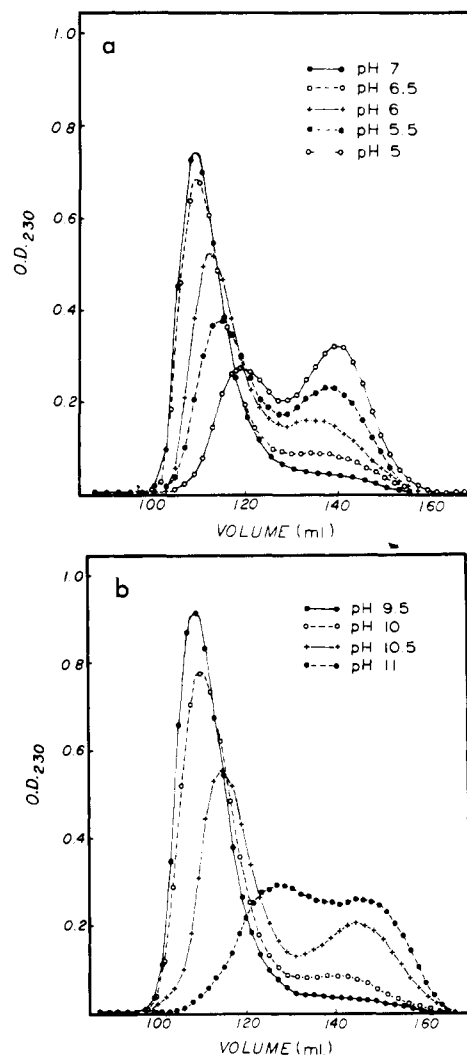


FIGURE 7: Small-zone gel filtration technique to determine octamer stability as a function of pH. Each profile represents the elution of 2 mL of the purified octamer (2 mg/mL) on G-100 columns equilibrated at 4 °C in 2 M NaCl at the indicated pH. (a) pH range 5–7 using 20 mM NaH_2PO_4 as buffer. (b) pH range 9.5–11 using 10 mM Tris-HCl, 10 mM Na_2HPO_4 as buffer.

the major reason for the controversy over the molecular weight of the core complex, is the failure to isolate the complex in a *purified* form. In previous reports, the complex defined as “core complex” has simply been that material which is extracted with 2 M NaCl from chromatin prewashed with 0.6 M NaCl (Campbell & Cotter, 1976; Lilley et al., 1977; Thomas & Butler, 1977). Polyacrylamide gels demonstrate that all four core histones are present in this extract in approximately equal ratios, but cannot offer information on the state of assemblage of these polypeptides, i.e., whether they are associated into a single complex. Indeed, the sedimentation velocity experiments presented in these reports suggest that significant amounts of lower molecular weight complexes as well as aggregated material are present in these extracts (Lilley et al., 1977; Thomas & Butler, 1977). Weintraub et al. (1975) did attempt to purify the core complex. Their complex, however, did not migrate as a sharp peak on gel filtration columns and exhibited substantial degrees of dissociation and aggregation on sucrose gradients. All experiments described in the present study were conducted with *purified* core complex. This complex migrates as a sharp peak on G-100 columns or sucrose gradients with no indication of aggregation or irreversible dissociation (Figure 2).

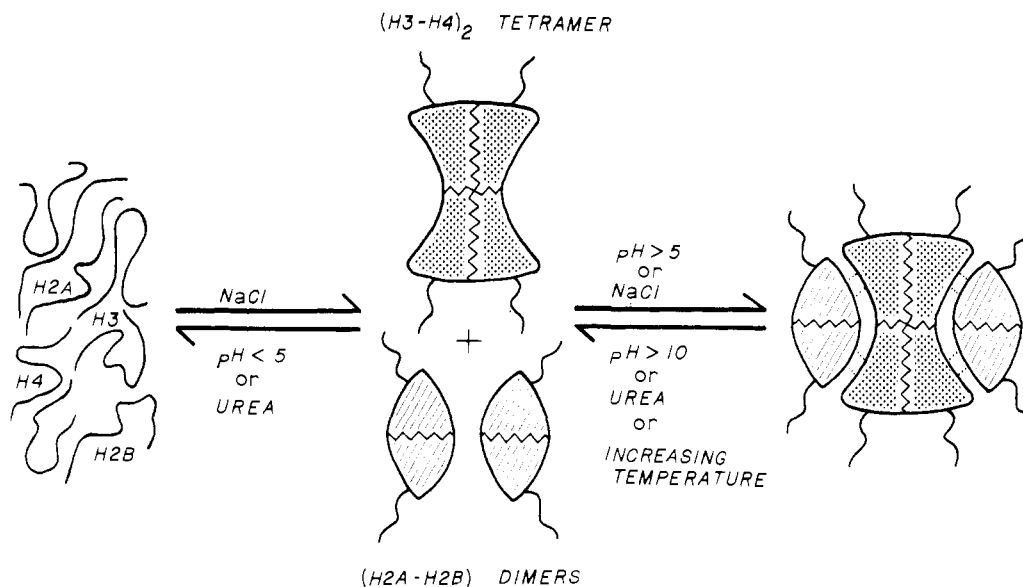


FIGURE 8: Self-assembly of the core histone complex. The system is depicted in a freely reversible equilibrium with the last transition showing a strong dependence on concentration due to the weakness of the forces stabilizing the octamer. Environmental parameters demonstrated to affect the state of the equilibrium are ionic strength, temperature, and pH in the manner indicated. The eight polypeptides comprising the complex self-assemble first into two distinct types of quaternary structures, i.e., the H2A-H2B dimer and the (H3-H4)₂ tetramer. These oligomeric structures are believed to associate primarily via extensive hydrophobic interactions (symbolized by zig-zag lines) and are quite stable. They enter into a higher order of assembly involving two H2A-H2B dimers and one (H3-H4)₂ tetramer to form the octameric core histone complex. The forces regulating this last transition are weak and involve a limited number of contacts between the dimer and the tetramer subunits (symbolized by dotted lines). We propose that these forces are derived primarily from hydrogen bonding, since the octamer is disrupted by small increases in temperature, or by low concentrations of urea, and is stable within a pH range (7-10) with sharply defined boundaries.

The possibility that the histone octamer reported here is actually derived from the association of two "heterotypic tetramers" (one each of the four core histones), as recently suggested by Chung et al. (1978), is not supported by our data. In the second half of this report, various parameters were studied which affected the stability of the histone octamer. In all cases where the association constant of the octamer was lowered (Figures 4-7), there appeared to be a gradual shift in the apparent molecular weight of the core complex peak in the direction of lower molecular weight, corresponding to a decrease in the amount of H2A and H2B found under the peak. Such a gradual decrease in the relative abundance of H2A and H2B in the core protein peak is not consistent with the presence of a "heterotypic tetramer" in equilibrium with an octamer, but is consistent with the presence of an intermediate hexamer species. D'Anna & Isenberg (1974) have postulated that the H3-H4 tetramer in solution is not in equilibrium with an H3-H4 dimer; rather, their data best fit a monomer-tetramer equilibrium system with a very high association constant. It would appear unlikely, therefore, that the much weaker interaction of the H2A-H2B dimer with the H3-H4 tetramer as shown in the present report would be sufficient to dissociate the H3-H4 tetramer into dimers. It should be mentioned, however, that these studies have been conducted with histone complexes in the absence of DNA. It is conceivable that in the presence of DNA, quantitative changes occur in the association properties of the various histone complexes which might generate qualitatively different structures. We have no direct evidence to suggest that the histone octamer *when bound to DNA* cannot split into symmetric halves, each containing a "heterotypic tetramer" as described in the model of Weintraub et al. (1976).

Based on the core complex stability studies described in the second half of this report, we offer a scheme—diagrammatically illustrated in Figure 8—to explain both the self-assembly of the core histone complex and its structure in solution.

The eight polypeptides that constitute this complex assemble

into two successive states of organization, each controlled by unique sets of interactions. The first step of assembly results in the formation of the H2A-H2B dimer and the (H3-H4)₂ tetramer. The interactions responsible for the formation of these two quaternary structures extend primarily over hydrophobic segments of the polypeptides involved (indicated by the zig-zag lines in Figure 8). These interactions are quite stable since they require extreme experimental conditions for disruption. Bradbury & co-workers have suggested that residues 31-95 of H2A and 37-114 of H2B are involved in the tertiary structure of the H2A-H2B dimer, while residues 42-120 of H3 and 38-102 of H4 are involved in the formation of the H3-H4 tetramer (Moss et al., 1976a; Böhm et al., 1977). These regions involve most of the areas of the histone molecules that are not believed to be associated directly with the DNA.

The second step of assembly is between two H2A-H2B dimers and one (H3-H4)₂ tetramer and results in the formation of the histone octamer. The interactions responsible for this step are weak and involve, primarily, a limited number of hydrogen bonds between specific segments of the H2A-H2B and the (H3-H4)₂ subunits (indicated by the dotted lines of Figure 8). This suggestion is novel and is supported by the results of the present study on the effects of temperature, urea, and pH titration on the dissociation of the octamer.

Assuming that the histone interactions found in 2 M NaCl, pH 7.5, are similar to those found in chromatin, with the high concentration of NaCl merely substituting for the charge neutralization normally offered by the DNA, we suggest the following hypothesis concerning the role of the various histone complexes in the formation of chromatin. First, the tightly complexed H3-H4 tetramer and H2A-H2B dimer are the basic histone units bound to DNA. Association of these complexes with the DNA results in the primary left-handed supercoiling of the DNA helix in the nucleosome. Energy for this coiling could be derived from either the dehydration of the DNA grooves as a result of histone binding (Eickbush &

Moudrianakis, 1978) and/or the free energy of interaction between DNA-bound histone pairs (Camerino-Otero & Felsenfeld, 1977). That the interactions *between* the H2A-H2B dimer and the H3-H4 tetramer while bound to DNA are weak is supported by the body of evidence which indicates that, under a variety of conditions, H2A and H2B have similar extraction properties from chromatin which are distinctly different from the extraction properties of H3 and H4 (Ilyin et al., 1971; Kleinman & Huang, 1972; Bartley & Chalkley, 1972). More recently, it has been demonstrated that H3 and H4 cannot bind to DNA in a meaningful manner unless given the opportunity to form an H3-H4 unit in solution (Rubin & Moudrianakis, 1975). The presence of the H2A-H2B dimer did not significantly aid in the association of the H3-H4 complex with the DNA. Based upon these studies, we have reported a model of chromatin structure in which two turns of the supercoiled DNA helix are held together by the interaction between H3 and H4 to form a nucleosome (Moudrianakis et al., 1977). H3 and H4 in the absence of the other histones have indeed been shown to be capable of inducing a compaction in DNA which has chromatin-like properties (Boseley et al., 1976; Camerino-Otero et al., 1976; Camerino-Otero & Felsenfeld, 1977; Bina-Stein & Simpson, 1977).

It is generally believed that, while virtually all genomic sequences have a normal complement of histones, which give rise to the 200 base pair repeats found in nuclease studies, nucleosomes which cover transcriptionally active regions of DNA are in a different conformation than nucleosomes on inactive regions (Weintraub & Groudine, 1976; Garel & Axel, 1976). The results of the present report, demonstrating that the association of the H3-H4 tetramer with the H2A-H2B dimer to form the octamer is relatively weak involving hydrogen bonds, suggest an interesting mechanism by which subtle changes in the structure of nucleosomes may be regulated. Hydrogen bonds offer a high degree of specificity, and since they involve only a limited number of amino acids, they can easily be regulated by the cell. Blockage or formation of these hydrogen bonds would result in changes in the formation and stability of nucleosomes, while not affecting the basic association of the H3-H4 tetramer and H2A-H2B dimer with the DNA. These subtle changes in nucleosomal conformation could play a role in the determination of the accessibility of chromatin to the variety of macromolecules involved in the transcription or synthesis of DNA. We have suggested that the hydrogen-donating residues for these hydrogen bonds are either lysine or tyrosine amino acids. It is interesting to note, therefore, that the modification of lysine residues by acetylation or methylation is known to occur in chromatin (Louis & Dixon, 1972; Ruiz-Carrillo et al., 1975). While the predominant location of such modifications at the N-terminal ends of histones has suggested that these modifications result in altered histone:DNA interactions, it is an intriguing possibility that such modifications could also play a significant role in the histone-histone interactions regulating the accessibility of nucleosomes.

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